Universidade de Lisboa

Faculdade de Farmácia

Investigation of Flavonoids as inhibitors of human DNA topoisomerase IIα

Marta Raquel Fernandes Vicente

Mestrado Integrado em Ciências Farmacêuticas

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Final Report

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I - Resumo

O cancro, conhecido como a "doença do século", é considerado um dos maiores problemas de saúde pública a nível mundial cuja incidência tem aumentado nas últimas décadas prevendo-se que progrida nesse sentido.

Ainda que os avanços na medicina tenham possibilitado um conjunto de opções de tratamento que reduzem significativamente as consequências desta doença, a quimioterapia é um dos principais métodos no combate ao cancro. No entanto, além das inúmeras reações adversas associadas à quimioterapia, existem mecanismos de resistência do cancro a esses fármacos. Assim, torna-se imprescindível a procura de novas moléculas anticancerígenas com um nível de atividade promissor e elevado índice terapêutico.

Os flavonoides são um grupo de metabolitos secundários obtidos das plantas capazes de promover um grande número de benefícios para a saúde, devido aos seus efeitos anti-inflamatórios, anti-oxidantes, anti-depressivos, hepatoprotetores e anti-carcinogénicos.

O seu papel principal prende-se com a capacidade de induzirem mecanismos apoptóticos e diminuírem a proliferação das linhas celulares tumorais. De acordo com vários estudos, alguns dos seus efeitos são mediados através da inibição de uma classe de enzimas que se intitulam de DNA topoisomerases.

As DNA topoisomerases são uma grande família de enzimas que participa nos processos de reparação, replicação, transcrição, recombinação, segregação e ainda condensação do DNA cromossomal. Existem duas classes de topoisomerases: tipo I e tipo II.

Este estudo focou-se na topoisomerase eucariótica humana do tipo II, na sua isoforma α, que é dependente do ciclo celular, da proliferação celular e aparenta ter uma grande distribuição em todos os tipos de tecidos.

As topoisomerases IIα originam intervalos transitórios na dupla cadeia de DNA, em condições normais e em concentrações equilibradas pela célula. No entanto,

concentrações elevadas de topoisomerases levam a intervalos permanentes, o que constitui um perigo por citotoxicidade e numerosas mutações, induzindo a morte celular.

Existe uma grande diversidade de agentes capazes de inibirem a proliferação de células neoplásicas, podendo ser divididos em duas grandes classes: os "inibidores catalíticos" ou os "venenos de DNA".

Numerosos estudos demonstram que os flavonoides exibem atividade anticancerígena e podem fazê-lo comportando-se tanto como venenos de DNA ou como inibidores catalíticos. Este projeto teve como objetivo contribuir para a clarificação dos mecanismos de atuação da topoisomerase IIα enquanto possíveis agentes anticancerígenos, de modo a poderem ser incluídos em novas terapias.

Para tal, iniciou-se este estudo através de uma análise de nove flavonoides, previamente selecionados por modelação molecular. Realizou-se um "screening" virtual através de dois métodos: o docking molecular, que representa uma análise ao composto baseada na sua forma de ligação, e ainda uma análise de farmacóforos, baseada na estrutura do composto, utilizando para tal os programas GOLD e LigandScout, respetivamente.

Utilizaram-se vários métodos bioquímicos que nos permitiram investigar o mecanismo de inibição dos compostos na enzima topo IIα, nomeadamente: ensaio de relaxamento com triagem de alta produtividade; ensaio de ressonância de plasmon de superfície; ensaio de decatenação e o ensaio de clivagem.

Estes ensaios permitiram concluir que os flavonoides se ligavam ao domínio de ATP da enzima topo IIα, e que cinco dos nove compostos conseguiam, efetivamente, inibi-la, tendo sido os compostos **2** e **7** mais potentes do que o etopósido, de referência.

Embora estes resultados tenham contribuído de forma significativa para a compreensão dos mecanismos catalíticos das topoisomerases, existem muitos aspetos que ainda permanecem desconhecidos.

Palavras-chave: Cancro; Agentes Anticancerígenos; Flavonóides; DNA Topoisomerase IIα humana; Design de fármacos.

II - Abstract

Cancer has been considered a major public health problem worldwide, which has increased in incidence in recent decades and is expected to further progress in occurrence in the future. Although advances in medicine have enabled a wide range of treatment options that significantly reduce the consequences of this disease, chemotherapy remains one of the primary techniques to be used in the fight against cancer. As this approach is strongly associated with drug resistance and side effects, the search for new anticancer molecules with a promising level of activity and high therapeutic index is essential.

DNA Topoisomerases are a large family of essential enzymes that participate in the repair, replication, transcription, recombination, segregation and even condensation processes of the chromosomal DNA. There are two main classes of topoisomerases known as Type I and Type II. The subject of our research was the human topoisomerase type II in its α isoform, as it is upregulated in fast growing cells, including cancer cells.

There is a variety of agents capable of inhibiting human type II topoisomerase, which can be divided into two broad classes. The first is the group of more established topo II poisons, which are widely used in therapy and are very efficient in treating several types of cancer. However, they suffer from several severe side effects such as cytotoxicity, induction of secondary tumours as well as resistance to existing topo II-based therapies. To overcome this problem, the second diverse group of catalytic inhibitors is being actively developed that tries to overcome the limitations of this established group.

In this work, we used the results of a previously performed large-scale pharmacophore-based virtual screening of a library of natural products, which considered the topological characteristics of some of the previously discovered inhibitors that target the topo IIα ATP binding site. The screening identified nine natural compounds from the Flavonoids chemical class with potential ability to inhibit the topo IIα.

In the initial HTS topo II relaxation assay, we identified new five flavonoids with topo II inhibition activity with some compounds possessing this activity in low micromolar

range. Using these data, we also obtained preliminary SAR information for this class of compounds.

Subsequently, we performed molecular docking calculations and devised 3D structure-based pharmacophore models to study the molecular interactions between the natural products that were discovered by our pharmacophore-based virtual screening and the targeted human topo IIα ATP binding site.

With selected potent flavonoids (2 and 7), we tried to gain more insights into their inhibition mechanism. Using topo IIα decatenation and cleavage assays, we showed that these selected natural products act as potent catalytic inhibitors. Next, surface plasmon resonance (SPR) experiments were performed for all active compounds suggested binding to the isolated topo IIα ATPase domain where the ATP binding site is located. However, the ligand recognition process appeared more complex that pure 1:1 binding to this binding site.

The results obtained could potentially provide new information about these lead compounds of natural origin that could pave the way to further development of novel anticancer agents exploiting the catalytic topo IIα inhibition paradigm.

Keywords: Cancer; anticancer agents, natural products flavonoids; human DNA topoisomerase IIα; drug design.

III – Acknowledgments

After this incredible journey I couldn't be more thankful for all the experience I have gained during Erasmus programme. All the people I have met, they somehow have changed my personality and made it better. This was, sincerely, one of the best parts of being student. To study abroad.

Firstly, I would like to thank to my supervisor Dr. Andrej Perdih for receiving me so warmly in Slovenia, to including me so well in the team and, of course, for guiding me and teaching me during this project. Also, I am grateful to all the support that Kaja Bergant gave me, to her patience and her availability to every question I had along my research.

Professor Doctor Maria Umbelino for her scientific co-orientation, for all the advises and comments in my thesis and mostly for her motivation.

This master thesis represents a great accomplishment over these five years and surely these people played a key role for me.

Now I want to express in writing all my deep thanks to my parents and boyfriend, because they stood beside me no matter what and they have been my strength and my inspiration in life. Thank you for all the conversations and for being my refuge in everything.

Finally, I would like to express my gratitude to my best friends, for all the moments together, for the companionship and the true meaning of the friendship and especially for not only applauding my conquests but also appeasing my sorrows.

I am proud to becoming a Pharmacist and I hope to be a good one, and throughout my professional life I hope remembering all the values that FFULisboa has taught me. I am feeling fulfilled and I can't wait to start a new chapter in my life!

Lisbon, November 2019

Marta Vicente

IV - Acronyms

ATP – Adenosine triphosphate

ADP – Adenosine diphosphate

DNA – Deoxyribonucleic acid

IC_{50} – Half maximal inhibitory concentration

SPR – Surface plasmon resonance

kDa – Kilodalton

kDNA – kinetoplast DNA

Gyr – Gyrase

Topo – Topoisomerase

KD – Dissociation constant

PDB – Protein Data Bank

EDTA – Ethylenediamine tetra acetic acid

BSA – Bovine serum albumin

TWEEN – Polysorbate

DTT – Dithiothreitol

Tris-HCl – Tris Hydrochloride

HEPES – (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

RMSD – root-mean-square deviation

AMP-PNP – Adenylyl-imidodiphosphate

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1. Introduction

1.1 Cancer

Cancer is considered a major health problem in the world and it is expected to increase more and more with the approximately 20,3 million new cases reported by 2030 (1). It is also one of the leading causes of death worldwide.

This pathological state occurs when a group of cells divide and grow in an abnormal way becoming independent from the control of the organism. There are different signals that dictate uncontrollably if the cells start either division pathway, differentiation or even apoptosis way, which can lead to different types of cancer (2).

In spite of the fact that for some types of cancer the mechanism of their onset remains unknown, there are some risk factors that can importantly contribute to its development such as excess body weight, smoking, and non-modifiable factors, such as genetic ones (3). It is well known that normal cells transform themselves into cancerous cells due to successive mutations inside the DNA genome which can produce proteins that can disrupt a delicate cellular balance. This can lead to fatal cells proliferation and can culminate in the development and tumor spreading (2).

Since 1940s antineoplastic drugs are used in the treatment of cancer and chemotherapy (utilization of one or more anti-cancer drugs) has successfully provided means to achieve remission from this disease or even complete recovery. It's the principal treatment of cancer for most patients along with the surgical removal of the cancer tissue. However, during such treatments numerous patients suffer from severe adverse reactions, with side effects such as hepatic, renal, cardiac, pulmonary, dermal and immunotoxicity toxicity, mostly due to non-selective way of actions of the majority of chemotherapeutics (4).

Thus, it is urgent to discover new drugs with fewer side effects and improved selectivity profiles that would result in less nonspecific toxicity. In addition to the development of new standard anticancer agents, new diagnostic methods (assays for tumor markers),

innovative therapeutic approaches and strategies (e.g. development of biological therapeutics) that better predict the cell behaviour play an important role for further successful tackling of this disease. There is a variety of targeted therapeutics influencing different "hallmarks" associated with cancer onset and development that were defined by Hanahan and Weinberg and are schematically shown in **Figure 1**(5).

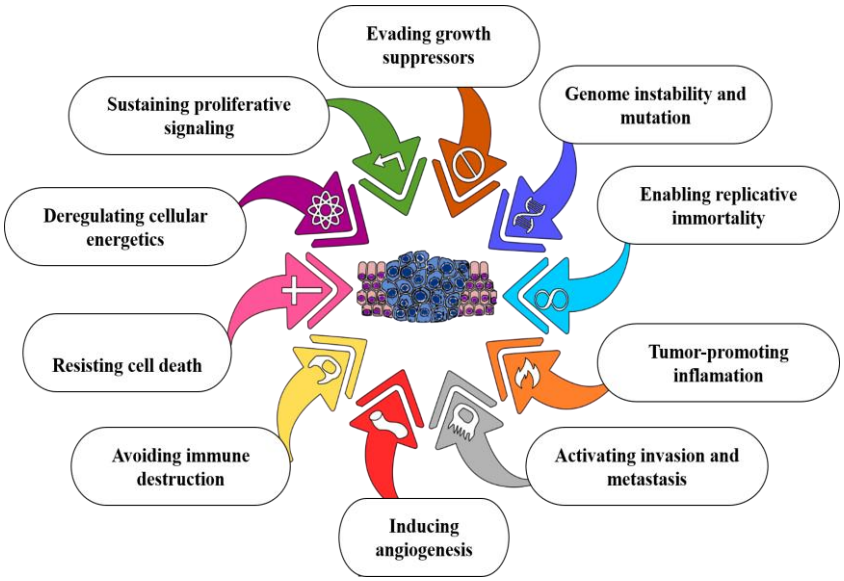


Figure 1 "Hallmarks of cancer" therapeutics are necessary for cancer development (5)

All these hallmarks of cancer are regulated by numerous complex signalling pathways. Hopefully, new research will eventually lead to the discovery of specific targeted therapeutic agents capable of shutting off that key pathway in a particular tumor (5).

Tumor progression is a succession of clonal expansions triggered by mutant genotype or even non-mutational changes affecting the regulation of genes. This leads to DNA-maintenance machinery (where topoisomerases fit) defects and vigilance failure which forces damaged cells into apoptosis. Concerning the hallmarks related to genome instability and mutation, DNA topoisomerases are well characterized targets and quite prevalent regarding their applications in cancer therapy (5).

1.2 DNA Topoisomerases – Established anticancer targets

It is known that the DNA molecule is highly compacted into the cell's nucleus due to its considerable length (around 3 m long). This is possible because of the involvement the scaffolding proteins which help to form a coiled and condensed structure named chromosomes (6).

Cells can control the compactness of the DNA in order to regulate the gene expression and despite the enormous compaction, DNA can be rapidly accessible to a group of processes like replication, transcription, recombination (6,7) and its repair (8). By considering the properties of the DNA's structure, it is evident that DNA's strands must be unlinked to make such topological changes possible.

The solution that nature produced to enable efficient topological changes of the DNA molecule is a class of ubiquitous enzymes called DNA topoisomerases which have been discovered, in 1971, by Prof. James C. Wang at Harvard University, USA (9–11).

DNA Topoisomerases can be divided into two major classes based on their mechanism of action: monomeric type I and multimeric type II. Type I enzymes act by generating a transient single-stranded break within one DNA double helix, while type II topoisomerase breaks one full double-stranded DNA strand, this forming a transient break allowing for a passage of another double-stranded DNA segment (8,12,13).

These enzymes can maintain the genomic integrity during these processes because they form a short-lived covalent bond between the enzymes´ tyrosyl residues and the terminal DNA phosphates during the cleavage reaction and every topoisomerases have this linkage as its specific characteristic (7,14). Both two types have further subclassifications, as shown in **Table 1** (12), and their subtypes are used to distinguish families that have distinct amino acid sequences, activities and/or structures (7).

Table 1 General division of the DNA Topoisomerase Subfamilies (12)

Subfamily	Topoisomerase	Species	Mechanism of Action
IA	Bacterial DNA Topoisomerase I	*Escherichia coli*	Single-stranded break creation
	Bacterial DNA Topoisomerase III	*Escherichia coli*	
	Yeast DNA Topoisomerase III	*Saccharomyces cerevisiae*	
	Mammalian DNA Topoisomerase IIIα	*Human*	
	Mammalian DNA Topoisomerase IIIβ	*Human*	
	Bacterial and archaeal reverse DNA gyrase	*Sulfolobus acidocaldarius*	
	Bacterial reverse DNA gyrase	*Methanopyrus kandleri*	
IB	Eukaryotic DNA Topoisomerase I	Human	Single-stranded break creation
	Poxvirus Topoisomerase	Vaccinia	
	Bacterial DNA Topoisomerase V	*Methanopyrus kandleri*	
IC	Archaeal DNA Topoisomerase V	*Methanopyrus kandleri*	Single-stranded break creation
IIA	Bacterial DNA gyrase	*Escherichia coli*	Double-stranded break creation
	Bacterial DNA Topoisomerase IV	*Escherichia coli*	
	Yeast DNA Topoisomerase II	*Saccharomyces cerevisiae*	
	Mammalian DNA Topoisomerase IIα	Human	
	Mammalian DNA Topoisomerase IIβ	Human	
IIB	Archaeal DNA Topoisomerase VI	*Sulfolobus shibatae*	Double-stranded break creation

Some of the type I and II topoisomerases are the best studied human topoisomerases. The main difference between them is the fact that type II topoisomerase are capable of removing the negative or positive DNA supercoils, and unlinking intertwined pairs of the newly replicated chromosomes, which type I topoisomerase are not able to (15).

1.3 Human DNA Topoisomerase IIα

Eukaryotic type II topoisomerase enzyme is a homodimer protein with the molecular mass of around 160 -180 kDa. It enables the DNA topological changes via a multiple step catalytic cycle- that requires the hydrolysis of two ATP molecules in the presence of Mg^{2+} ions (8,12,16). Mammalian cells have two topo II isoforms - alpha and beta (Topo IIα and Topo IIβ). Alpha isoform is considered a primary target for cancer therapy because it is overexpressed in proliferating cancer cells in comparison to the beta isoform (12,17,18) and it relaxes the positively supercoiled plasmids faster than negatively supercoiled ones which makes it an optimal target for cancer therapy (12,19–21). Genetic studies had shown similarities in domain structures of both isozymes (22).

Inhibiting the function of the DNA topoisomerase IIα is a common and widely accepted approach exploited by cancer therapies. Since eukaryotic type II topoisomerases can be

well conserved, it is possible to get some structural insights about their structure and function from the topo II enzyme isolated from the yeast *Saccharomyces cerevisiae* where a full crystal structure has been solved and the findings then extrapolated to the human enzyme, leading to a composite picture (23–25) presented in **Figure 2** (13).

Human topoisomerase II is , similarly to its yeast counterpart, a homodimer and its structure can be divided into three dimer interfaces based on the homologies with the bacterial DNA gyrase, one at its N-terminus, one in the middle, and the other at the C-terminus (12,13,25).

N-terminal fragment is homologous to the B subunit of *E. coli* GyrB and it's composed of two subdomains: (13,26–28). The C-terminal fragment is homologous to the A subunit of E. *coli* GyrA and interestingly, its C-terminal tail has no homology with the DNA gyrase and contains amino acid sites that are involved in the phosphorylation with other proteins (12,13,29). Furthermore, the ATPase domain of topoisomerase II situated at the N-terminus, belongs to the GHKL (Gyrase, HSP90, histidine Kinase, mutL) superfamily of proteins.

The DNA binding gate located between the the N-terminal domain and the C-gate, being at the end of the C-terminal domain is comprised of three subdomains: Mg^{2+} binding TOPRIM (**Topo**isomerase/**Prim**ase) fold and a Winged Helix Domain (WHD) bearing the active-site tyrosine, and together they both play an important role in DNA segment cleavage, and the third is descibed as a "Tower" domain which interacts with the second monomer forming the full enzyme (8).

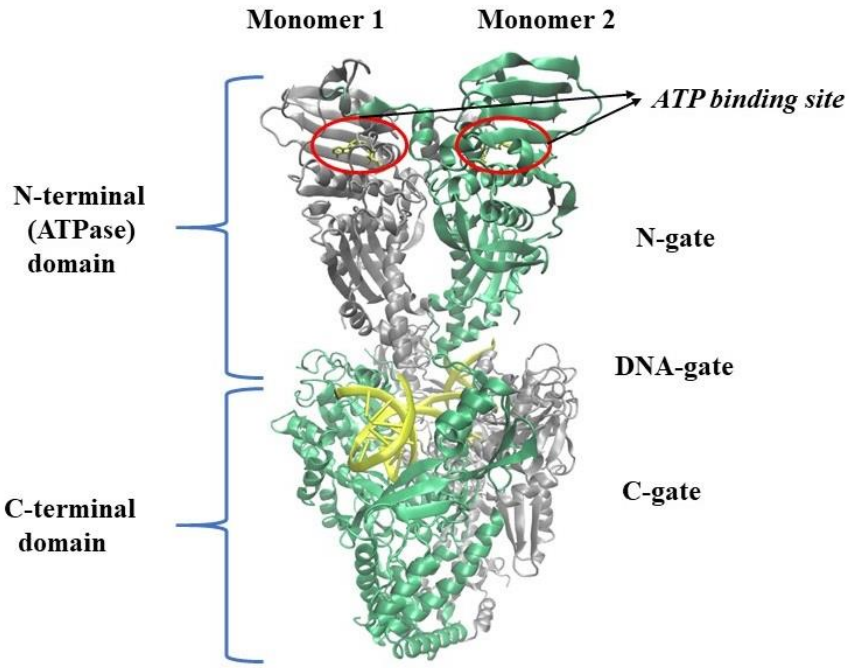


Figure 2. Structure of the full type II topoisomerase from *Saccharomyces cerevisiae* (PDB: 4GFH) (13)

1.3.1 The Catalytic Mechanism of the topoisomerase IIα – The catalytic cycle

Topoisomerase IIα catalytic cycle was first reported in the early 1990s and is schematically depicted in **Figure 3** (30), It is a multistep process in which it is assumed that the ATP hydrolysis triggers the topological changes by a so-called two-gate mechanism (31).

In the first step, one DNA molecule (chain A, also called the G ('gate')-segment) binds to the enzyme. Secondly, two molecules of ATP bind to the ATPase domain (N-terminal fragment), which causes its dimerization and capture of the second chain of DNA (chain B, also called the T-segment). Next, in the presence of the Mg^{2+} ions, the enzyme cleaves the chain A via a covalent binding of the two tyrosine residues from the winged helix domain on the 3' and 5' phosphate groups of the DNA chain A. This allows a formation of a transient short-lived covalent complex between the topo IIα enzyme and chain A. In

the fourth step, hydrolysis of the first ATP molecule occurs, consequently stimulating the passage of the DNA chain B through the chain A. Subsequently, chain A is relegated, and the C-terminal C-gate opens. In the sixth step the chain B is released and the C-gate closes. Finally, the second ATP molecule hydrolyses is accompanied by not only the release of chain A and ADP molecules but also the opening of the N-gate. So one full catalytic cycle is completed and the topoisomerase II is ready for the subsequent cycle (12,25,31–34).

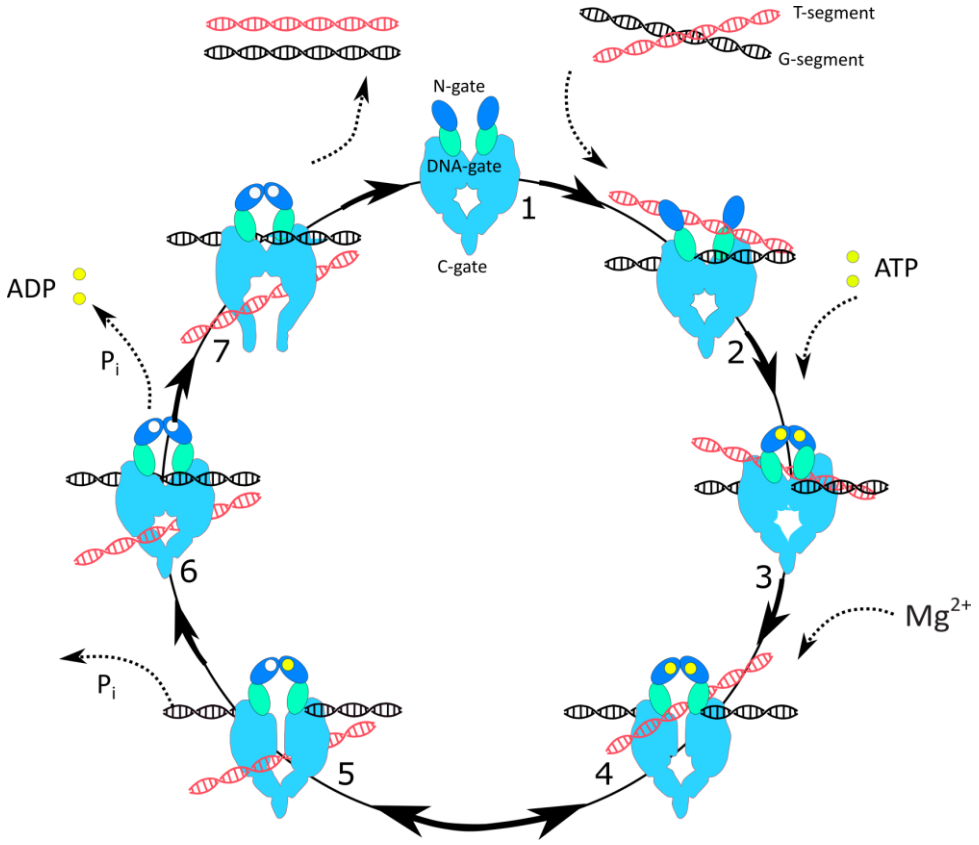


Figure 3. Scheme of DNA Topoisomerase IIα catalytic cycle (30)

As mentioned above, the DNA topoisomerase IIα levels are cell cycle-dependent and for that reason several studies have displayed that its levels may act as a solid marker of cell proliferation in tumors (35). Therefore, these structural information about the enzyme can be a valuable guide to discover inhibitors to interrupt different catalytic steps.

1.3.2 Human DNA topo IIα ATP Binding site – new target for anticancer therapy

As we will see in the next chapter, there are several ways how to inhibit the catalytic cycle of topo IIα. One of the new approaches is also to influence the binding of the ATP molecule. A major step forward in terms of enabling the structure-based drug design targeting the enzyme via this new potential mechanism was the determination of a crystal structure of the human topo IIα ATPase domain complex with a non-hydrolysable ATP analogue (AMP-PNP) and ADP ligand (36). The full structure of the human topo II has not been reported yet.

Figure 4 shows the overall structure of the ATPase domain form the human enzyme along with the topology of the ATP binding site which reveals that the adenine ring of AMP-PNP forms a hydrogen bond with the side chain of the carbonyl oxygen of the amino acid Asn120. Furthermore, the crystal water molecules and residues Thr215, Tyr34' are part of a wider H-bond network that act in the stabilization of the adenine ring. Two hydroxyl groups of the ribose sugar are stabilized via Ser149, and Asn150 H-bond interactions. The conformation of the AMP-PNP is finally firmly docked into the ATP binding site by such residues as Asn91, Lys168, Ala167, etc., which interact with oxygen atoms in the phosphate part (12).

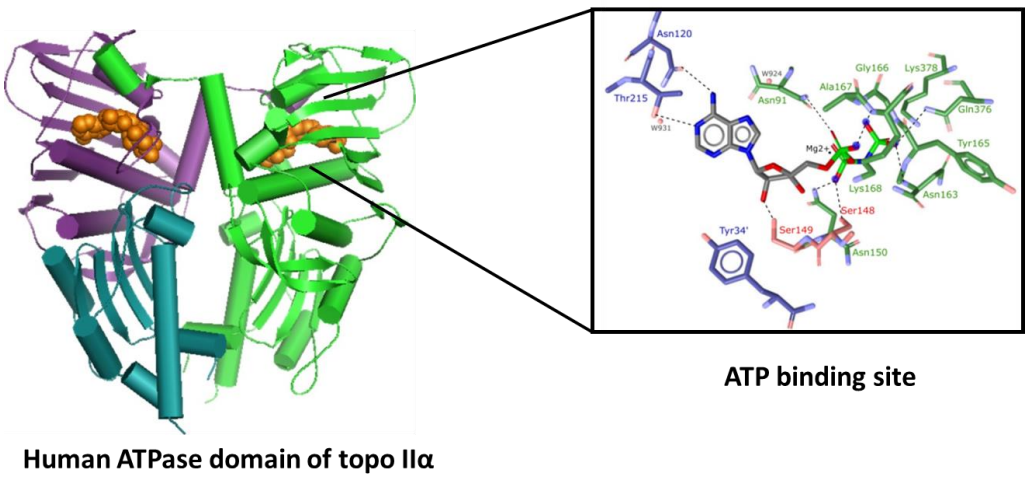


Figure 4. Structure of the human ATPase domain of topo IIα and topology of its ATP Binding (PDB: 1ZXM), LigandScout

This provided experimental information about the human topo IIα can greatly add in the structure-based drug design efforts, since it´s now possible to more precisely determine the topology and electrostatics of the potential ligands that might interact with this binding site.

1.4 Inhibitors of the human DNA Topoisomerase IIα

Human DNA topoisomerase IIα is one of the most established anticancer targets with several molecules already proficiently used in clinical practice. These topo IIα targeting agents are normally classified into two large groups, as presented in **Figure 5** (30), namely **catalytic inhibitors** and **topoisomerase II poisons.** Their characteristics diverge based on the type of inhibition mechanism they exhibit on this enzyme (6,12).

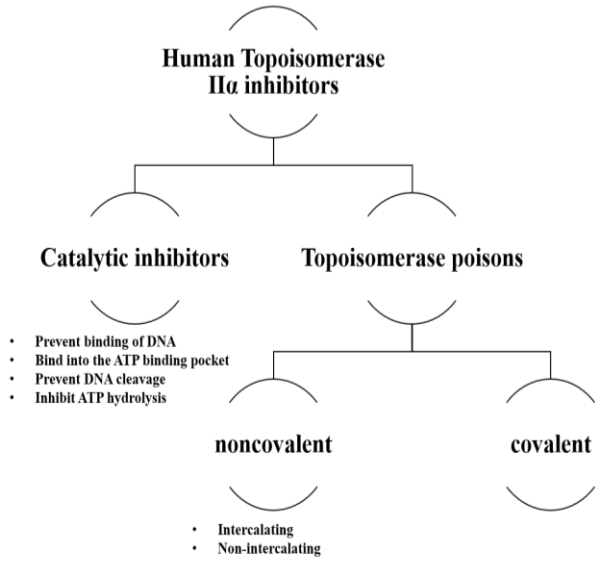


Figure 5. The general division of the human topoisomerase IIα inhibitors (30)

1.4.1 Topo II Poisons

Topo II poisons are the more established group of topoisomerase II inhibitors that contains several successful and potent anticancer drugs already used in therapy (34). They act by stabilizing of the covalent cleavage complex formed between the DNA and human topo IIα enzyme, which leads to permanent breaks in the cellular DNA, resulting in

chromosome translocations and apoptosis. This also converts this form of enzyme into a cellular toxin which is lethal to normal cells (13,30).

Topo II poisons can be further divided into intercalating or non-intercalating agents if they bind non-covalently (redox-independent) to the enzymes. Non-covalent group incorporates most of the drugs acting through this enzyme. There are a lot of the prominent examples as intercalating agents such as Doxorubicin [**1**], Daunorubicin [**2**], Mitoxantrone [**3**], Epirubicin [**4**] and Idarubicin [**5**] (see **Figure 6**) (30,34). Regarding to non-intercalating anticancer drug agents, Etoposide is the most known one (37).

Less known is the subgroup of the covalent topo II poisons (redox-dependent), which have not reached the market yet, including NAPQI (N-acetyl-p-benzoquinone imine) and Epigallocatechin gallate as some of the example molecules (13,37).

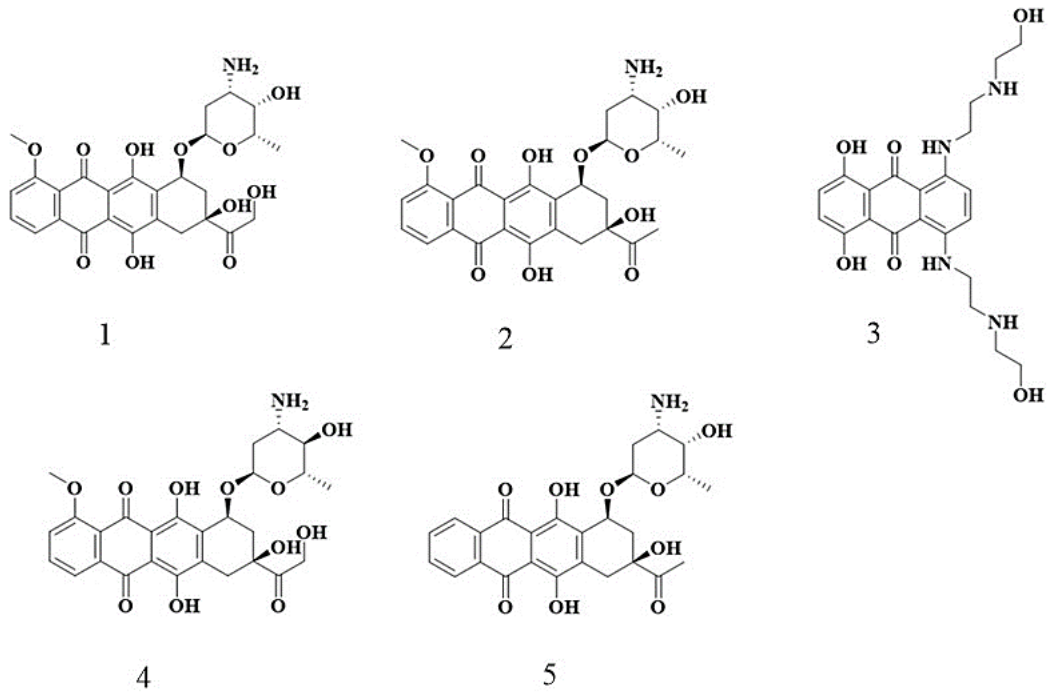


Figure 6. Selected examples of topo II poisons, most of them used in cancer therapy

In general, great number of antineoplastic drugs from the group of topo II poisons are widely used in cancer chemotherapy. However, this group, although very efficient, suffers from several side effects such as cardiotoxicity (34) and induction of secondary

malignancies. In addition, these drugs are also implicated in a type of Multiple Drug Resistance, called atypical MDR leads to a qualitatively modified topoisomerase II, less sensitive to the chemotherapy drugs. (38). Due to these limitations, further drug design efforts were initiated yielding novel promising compounds, in order to address these issues.

1.4.2 Catalytic Inhibitors of the human DNA topoisomerase II

Catalytic topoisomerase II inhibitors include structurally diverse groups of compounds that interfere predominantly in a single step of the topo II catalytic cycle and do not stabilize the covalent cleavage complex, as is the case for the group of topo II poisons (12,39,40).

They can be classified into four distinctive groups (see **Figure 7**): compounds that inhibit the ATP hydrolysis and trap the enzyme in a closed clamp, for example Dexrazoxane [**6**]; compounds that bind to the ATP binding site like Novobiocin [**7**]; compounds that prevent binding of the enzyme to the DNA like Aclarubicin [**8**]; and also a group of compounds that block DNA cleavage as Merbarone [**9**] (13,41–43).

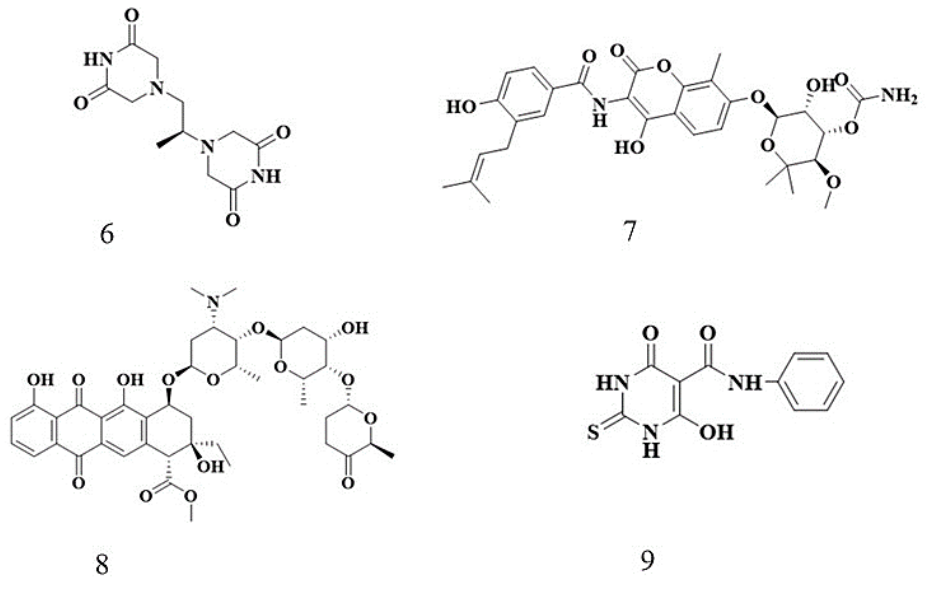


Figure 7. Selected examples of catalytic inhibitors of the human DNA topoisomerase IIα

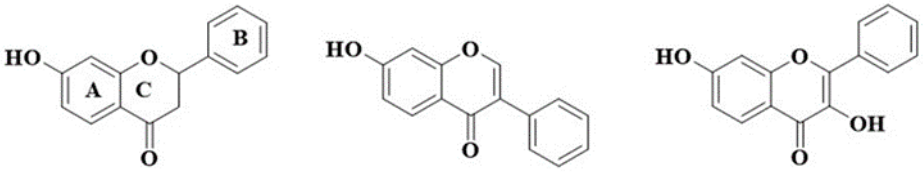
1.5 Natural products in topo II inhibitor research - Flavonoids

The therapeutic properties of plants have been widely recognised since the beginning of human civilization. Throughout history numerous pathological conditions have been treated using plant-derived medicines. Isolation and characterization of active natural products became an important aspect of drug design, either to serve to identify new hit/lead molecules or to use isolated molecules directly in the preclinical development.

Flavonoids are a diverse group of polyphenolic secondary metabolites -natural products- that can be found in human's diet as in the case of fruits, vegetables, legumes, and plant leaves namely beer, wine or tee, and mulberries, onions and apples too (44–46).

They comprise the most abundant natural source of antioxidants, as well as exhibit anti-inflammatory, antiallergenic and hepatoprotective effect. Some compounds from this group have also been reported to inhibit the DNA topoisomerase II, and for this reason, they are also linked to the prevention of cancer (38,47). However, even for the reported compounds the mechanism of action remains unclear.

Flavonoids are chemically phenyl-substituted compounds characterized by a 15-carbon basic structure (C6-C3-C6), composed of a (C6-C3) nucleus (the benzo ring A and the heterocyclic ring C), with a phenyl (the aromatic ring B) substitution usually at the 2-position (48). They have three major classes as illustrated termed flavones, isoflavones and flavonols (46) shown in **Figure 8** (44) Flavone examples: luteolin, apigenin, diosmetin; flavonol examples: myricetin, quercetin, fisetin and finally, isoflavone examples: genistein, biochanin A (44).



Flavone **Isoflavone** **Flavonol**

Figure 8. Structures of possible flavonoid core scaffolds (45)

The mechanistic basis for the physiological actions of Flavonoids is currently not very well understood. Published studies have shown that flavonoids could act both as topo II catalytic inhibitors or topo II poisons. Most of these studies have been conducted *in vitro* with purified DNA.

Therefore, further studies of other flavonoid compounds within the scope of this work could provide important information of advancing this aspect of anticancer development and could also help in clarifying the effects of these dietary agents on topoisomerases in order to understand their potential as well as toxicity.

1.6 Developing new drug molecules – The Drug design cycle

Drug Design cycle and computer-added molecular design enable a more efficient exploration of the available vast chemical space using modern computer technology. Very often the term molecular modelling can be used interchangeably with computational chemistry to describe the application of theoretical methods aiming to elucidate the mechanism of action of known drug molecules or to design and optimize new drugs (49).

Molecular modelling usually starts with the construction/utilization of a 3D molecular model of the selected target. Experimental data about the 3D structures of biological macromolecules mostly proteins are publicly available in the Protein Data Bank (PDB) (50).

Virtual screening is a term describing the automatic screening of large libraries of virtual or commercially available molecules that are carefully assembled taking into account proper geometry, protonation pattern, aromaticity, tautomer state and stereochemistry. The virtual molecule is treated as a hit molecule, by applying two major techniques or approaches: The ligand-based drug design (LBDD) that uses ligand information (pharmacophore models) to select the molecules, and further the structure-based drug design (SBDD) that uses information about the macromolecular target or ligand-target complexes (molecular docking) for compound selection (50,51).

Structure-based drug design is mostly connected with the method of molecular docking which determines the preferred conformation of the investigated molecule in the targeted

active site, based on the assumption that a stable complex between these two species can be formed. Several software programmes were developed that enable such computations, for example DOCK, FlexX, GOLD, AutoDock, etc., and the result of the molecular docking is a list of calculated protein-ligand complexes ranked according to the scoring function values that determine/assess their favourability (50,52).

In ligand-based approaches pharmacophore models are preferably used. Pharmacophore, is defined as an the ensemble of steric and electronic features of the ligand that are crucial for the optimal interatomic interactions with a specific biological target leading to the induction or inhibition of the biological response (50,53). Both molecular docking and pharmacophore modelling are integrated into a drug design cycle the process aimed to improve virtual screening for discovering novel active compounds, as depicted in **Figure 9**.

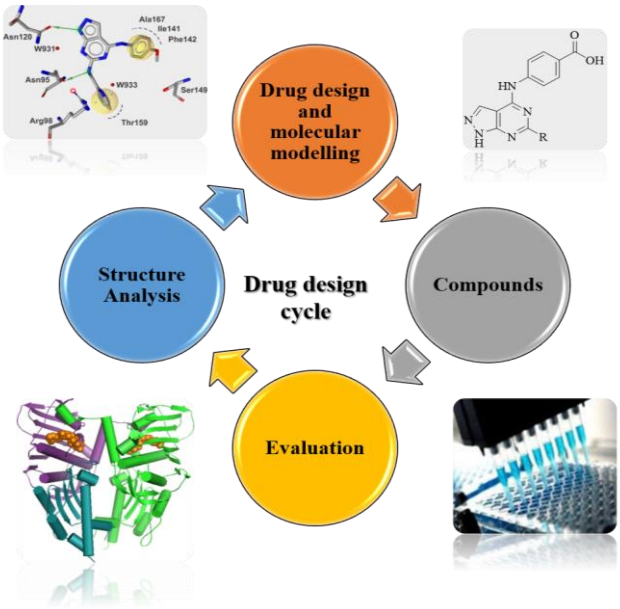


Figure 9. General scheme of the Drug design cycle – used in the design and evaluation of novel drug compounds.

The first step in the drug design cycle preferably starts with a known structure of the investigated macromolecular target. The goal here is to find compounds or fragments of

compounds positioned into selected regions of the targeted macromolecule by screening libraries of compounds using molecular modelling techniques. Based on their steric and electrostatic interactions with the interested target site, compounds are scored and ranked, and the best ones are tested through biochemical assays (54). If no target structure is available ligand-based approaches or other methods of computational chemistry such as homology modelling can be used.

Next step in the cycle comprises the synthesis of the selected compounds or acquisition of the available commercial molecules. These molecules are in the third stage evaluated in a series of biochemical and biophysical assays to determine the mode of action of these compounds. Finally, structure determination of the target in complex with a promising compound, that reveals possible optimization sites and the cycle is repeated.

After several cycles of molecular modelling, synthesis, experimental evaluation and structural studies , the active compounds usually show improvement in binding and, often, specificity for the target so that can enter more complex preclinical investigations (54).

2. Aims of this study

Human DNA topoisomerase IIα is an essential enzyme, which plays an important role in cellular machinery and is for this reason, a validated target in the development of anticancer drugs. It is also highly relevant for the development of new molecules of topoisomerase IIα inhibitors, especially due to the limitations imposed by severe side effects and cancer cell resistance associated with the group of clinically used topo II poisons.

Our work builds on the ongoing research aimed to discover and evaluate novel catalytic inhibitors of human topoisomerase IIα targeting the ATP binding site. It was performed at the National Institute of Chemistry (Kemijski inštitut) in Ljubljana, Slovenia in collaboration with the Faculty of Pharmacy at the University of Ljubljana.

This project started with a previously completed large-scale pharmacophore-based virtual screening of a library of natural products that identified 9 natural compounds from the flavonoid chemical class that could possess topo IIα inhibition potential. The derived pharmacophore model took into account the topological characteristics of some of the previously discovered inhibitors that target the topo IIα ATP binding site along with structural information of the native AMP-PNP ligand bound to this targeted area. Our work performed between February and April 2019 in the scope of the Erasmus+ program started with this information and was divided into two major parts the computational and experimental part, which substages are depicted in **Figure 10**.

In the computational part we performed molecular docking calculations and devised structure-based pharmacophore models to study molecular interactions between the natural products that were discovered by our pharmacophore-based virtual screening and the human topo IIα ATP binding site.

In the Experimental part, we used several experimental methods to assay and determine the properties of selected hit compounds in order to establish if they could inhibit the enzyme's activity and get more insight into their inhibition mechanism. In this part topo

II relaxation assay, topo II mediated decatenation and cleavage assays as well as surface plasmon resonance (SPR) methods were utilized.

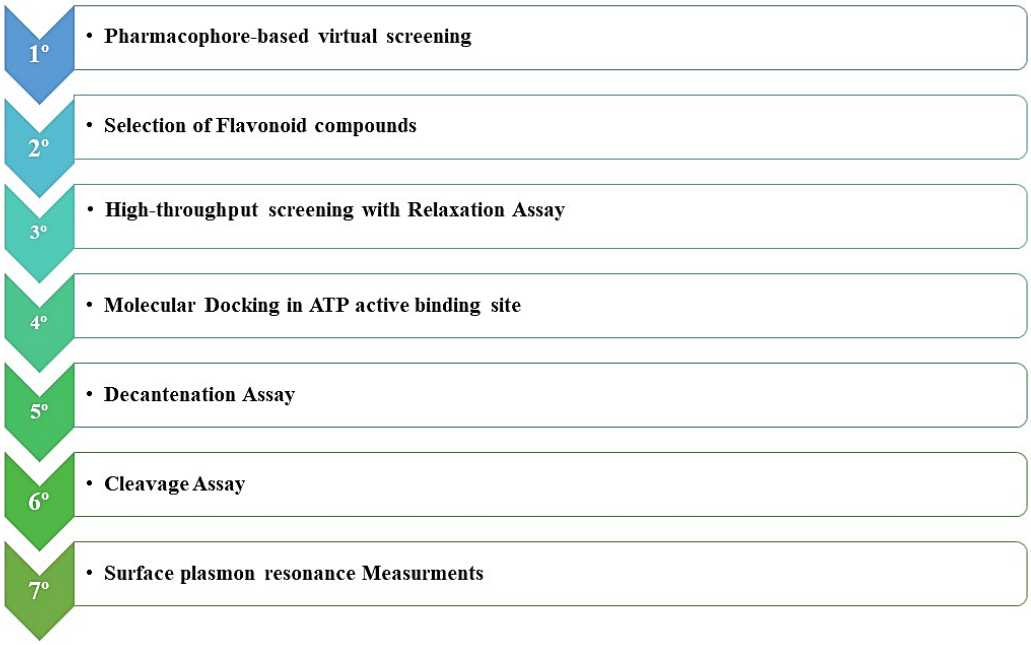


Figure 10. Schematic representation of the computational and experimental workflow used in our work.

This research activities could potentially lead to the discovery of new active agents capable of improving existing therapies in the treatment of cancer. In addition, we will also generate new information about the mode of action of flavonoid natural products when interacting with the topo II enzyme.

3. Computational approaches

3.1 Molecular Docking and Pharmacophore modelling calculations

Before docking experiments it is necessary to perform the validation of the used docking settings according to the recommended guidelines (55). Molecular docking experiments of the studied molecules **1**-**9** were performed using an established GOLD docking tool (56) and the available 3D structure of the human topo IIα ATPase domain (PDB code: 1ZXM) (25). The validation of GOLD docking tool was performed by redocking the native ligand AMP-PNP molecule into its binding site.

To summarize, the active site was defined as 10 Å radius around the reference ligand AMP-PNP and hydrogen atoms were added to the protein. Magnesium ion and all waters were removed except for crystal water molecules W924A and water W931A which were shown to play an important role in the molecular recognition in previous studies and were thus kept.

Docking calculations were performed by applying the following parameters of the GOLD genetic algorithm (GA): Population size=100, Selection pressure = 1.1, No. of Operations = 100000, No of Islands = 5, Niche size = 2, Migrate = 10, Mutate = 95, Crossover = 95. GoldScore scoring function was used.

Obtained binding pose of the docked AMP-PNP closely resembled the experimentally determined one, with the best RMSD agreement of 0.9 Å what indicated that used docking settings are reliable. The same scoring function and described docking settings were used for the molecular docking calculations of the active hit compounds.

Docking calculations were visualized using LigandScout software (57) where the structure-based pharmacophores of the obtained predicted ligand-topo IIα target protein complexes were derived using default settings of the pharmacophore recognition algorithm.

4. Experimental Methods

There are several biochemical methods available that allow us to probe the compounds' inhibition mechanism of topo IIα. The enzyme namely operates via a complex catalytic cycle and thus a combination of methods must be used (30).

The experimental methods used in this study are listed below. They will help us to assess first whether flavonoid hit compounds inhibit the targeted topo II enzyme and subsequently provide more details about its inhibition mechanism.

4.1 HTS relaxation Assay

Assay Principle

HTS topo IIα mediated relaxation assay is used as an initial screening tool to assess if these compounds are able to inhibit the normal process of topoisomerase II-catalysed relaxation on the supercoiled plasmid.

Topo IIα normally fully relaxes the supercoiled plasmid, but in the presence of the inhibitor, this process is blocked. The main principle behind this assay is that supercoiled plasmid can form a triplex DNA with oligonucleotides more readily than the relaxed one (30,58). The assay, however, cannot distinguish between the catalytic inhibitors and topoisomerase II poisons, so for more detailed characteristic of mechanism of inhibition, additional assays should be performed.

To briefly present the assay, first oligonucleotide is immobilized on the microwell plate (with streptavidin-biotin interaction). Then incubation of the topo II enzyme, supercoiled plasmid and oligonucleotide in the appropriate buffers occurs, to enable the formation of triplex. The medium is than changed, to remove the free plasmid. In the last step, the fluorescence stain is added, whose fluorescence increases, when bound to the formed triplex. The assays principle is illustrated in **Figure 11** (30) and was performed on black streptavidin-coated 96-well microtiter plate.

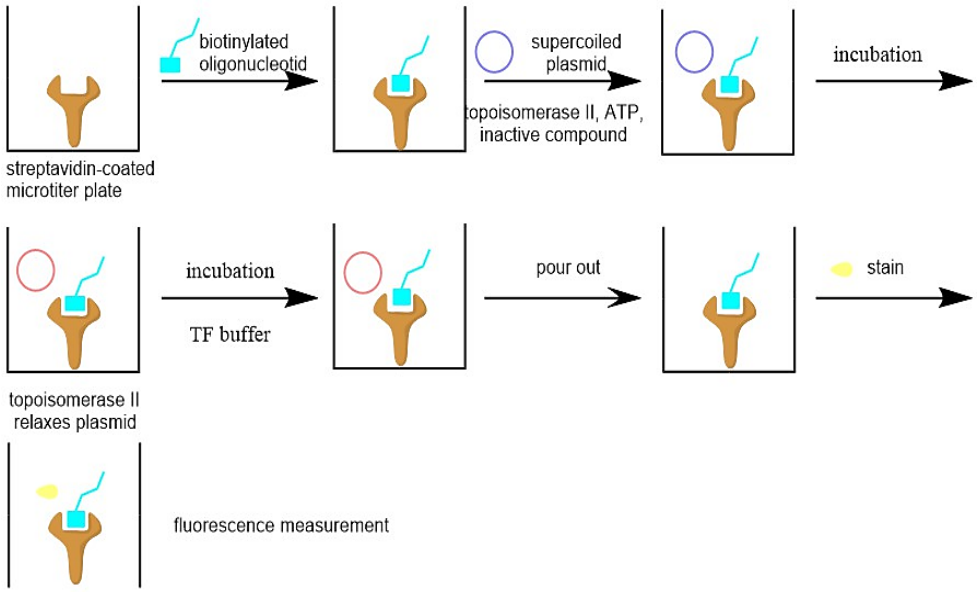


Figure 11. The principle of the HTS human topoisomerase IIα -mediated DNA relaxation assay (30)

Assay Details

After rehydration of the wells using Wash buffer (20 mM Tris–HCl (pH = 7.6), 137 mM NaCl, 0.005 % (w/v) BSA, 0.05 % (v/v) TWEEN-20®), biotinylated oligonucleotide was immobilized in the wells. The excess oligonucleotide was washed off with Wash buffer.

Next, the enzyme was incubated with the subtract (supercoiled plasmid pNO1) in reaction volume of 30 µL. Enzyme was diluted to appropriate concentration with dilution buffer (50 mM Tris-Hcl (pH = 7.5), 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 50 % (v/v) glycerol, 50 µg/mL albumin). Then 3 µL of tested compounds, diluted in 10 % DMSO, were added into the well, except for positive and negative control. Final concentration of DMSO was 1 %. Mixtures were incubated at 37 °C for 30 min, then TF buffer (50 mM NaOAc (pH = 5.0), 50 mM NaCl, 50 mM $MgCl_2$) was added to the wells and incubated at room temperature for additional 30 min to allow triplex formation To eliminate the aggregation and non-specific inhibition a surfactant (0.008% TWEEN-20®) was added in the reaction mixture. Unbound plasmid was washed off with TF buffer and stained with DNA-detection Dye in T10 buffer (10 mM Tris–HCl (pH = 8) and 1 mM EDTA). After mixing, fluorescence was read using Synergy Mx (Biotek) (Excitation: 495 nm and

Emission: 535 nm) (40). As positive control Etoposide was used. Assay was performed at the 2, 50, 100, 200 μM concentrations of compounds **1, 2, 3, 4, 5, 6, 9** and etoposide (positive control) and 0.05, 0.5, 5.0 and 50 μM concetrations for compounds **7** and **8**. IC_{50} values were calculated using Graphpad Prism 7.0 Software (59). The measurements were performed in duplicates.

4.2 Topo IIα-mediated DNA decatenation Assay

Assay Principle

Decatenation assay, depicted in **Figure 12** (30), is used to investigate if compounds can inhibit the topo IIα decatenation , catalysed by human topo IIα (60). It was performed in collaboration with Inspiralis (Norwich, UK).

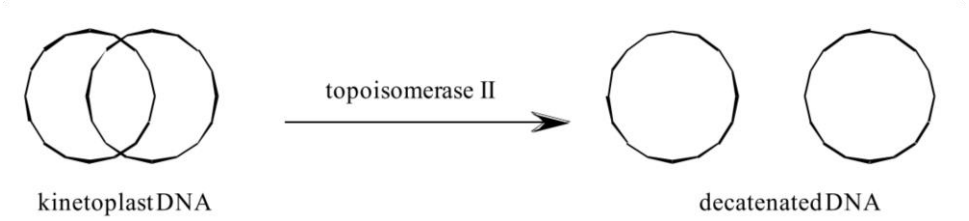


Figure 12. The general principle of DNA decatenation assay (30).

Assay Details

The substrate used in this assay was kinetoplast DNA (kDNA), mixed with topo IIα and investigated compounds in reaction buffer. For mixture electrophoresis was performed to observe moving of the DNA along the agarose gel. The main goal is to see if kDNA stays at the top of the gel, which tells us that the enzyme was inhibited and is thus unable to decatenate kDNA (30). One U of topo IIα was incubated with 200 ng kDNA in a 30 μL reaction at 37 °C for 30 minutes under the following conditions: 50 mM Tris HCl (pH 7.5), 125 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM EDTA, 0.1 mg/mL bovine serum albumin (BSA) and 1 mM ATP.

The reaction was then stopped by the addition of 30 μL chloroform/iso-amyl alcohol (26:1) and 30 μl Stop Dye (40 % sucrose (w/v), 100 mM Tris.HCl (pH 7.5), 10 mM

EDTA, 0.5 μg/mL bromophenol blue), before being loaded on a 1 % TAE gel run at 85 V for 90 minutes.

Bands were visualised by ethidium bromide staining for 15 minutes and destaining for 10 minutes. Gels were scanned using documentation equipment (GeneGenius, Syngene, Cambridge, UK) and inhibition levels were calculated from band data obtained with gel scanning software. (GeneTools, Syngene, Cambridge,UK). The assay was performed for compounds **2** and **7** at concentrations 0.05, 0.5, 5.0 and 10 μM and for etoposide (control) at concentrations 3.9, 31.5, 125 and 500 μM.

4.3 Topo IIα-mediated DNA cleavage Assay

Assay Principle

Human DNA topoisomerase IIα -mediated cleavage assay, depicted in **Figure 13** (30), is used to classify compounds as topoisomerase II catalytic inhibitors or topo II poisons (61) and it was performed in collaboration with Inspiralis (Norwich, UK). In the case of inhibition by topo II poisons the human topo IIα-DNA covalent complex is stabilized, leading to linear plasmids that can be detected on the gel.

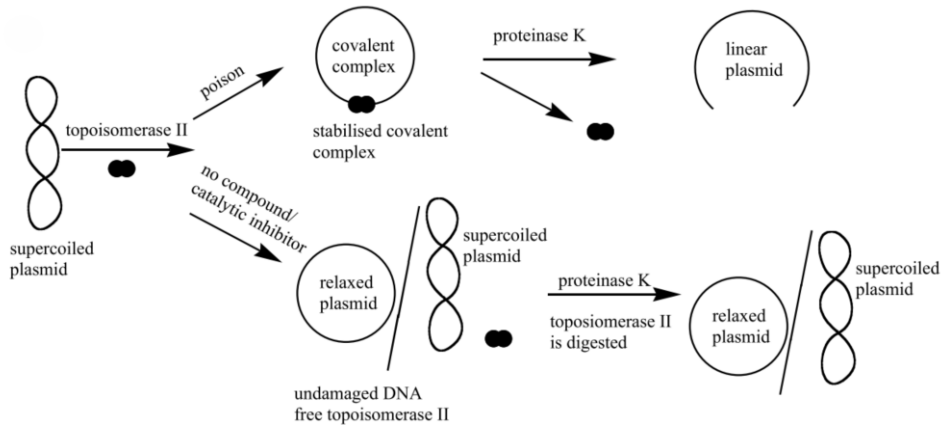


Figure 13. The general principle of the DNA cleavage assay (30)

Assay Details

One U of human topo II was incubated with 0.5 μg supercoiled plasmid DNA (pBR322) in a 30 μL reaction at 37 °C for 30 minutes under the following conditions: 20 mM Tris HCl (pH 7.5), 200 mM NaCl, 0.25 mM EDTA and 5 % glycerol. The reaction was then incubated for a further 30 minutes with 0.2 % SDS and 0.5 μg/μL proteinase K. The reaction was then stopped by the addition of 30 μL chloroform/iso-amyl alcohol (26:1) and 30 μl Stop Dye (40 % sucrose (w/v), 100 mM Tris.HCl (pH 7.5), 10 mM EDTA, 0.5 μg/ml bromophenol blue), before being loaded on a 1 % TAE gel run at 80 V for 2 hours. Bands were visualised by ethidium bromide staining for 15 minutes and destaining for 10 minutes. Gels were scanned using the documentation equipment (GeneGenius, Syngene, Cambridge, UK) and cleavage levels were calculated from the band data obtained with gel scanning software. (GeneTools, Syngene, Cambridge,UK). The assay was performed for compounds **2** and **7** at concentrations 0.05, 0.5, 5.0 and 10 μM and for etoposide (control) at concentrations 3.9, 7.8, 31.5, 125 μM.

4.4 SPR Measurements of ligand binding to the isolated topo IIα ATPase domain

Assay Principle

Surface plasmon resonance (SPR) experiments were used to determine if the tested compounds can bind to the isolated ATPase domain of the human topo IIα **(Figure 14** (62)**)**. A target molecule is anchored onto the gold-coated surface of a sensor chip and the underside of a glass carrier is irradiated with light. If they bind, the resulting change in mass on the gold surface can be registered and other parameters like association or dissociation rate constants can be measured (62).

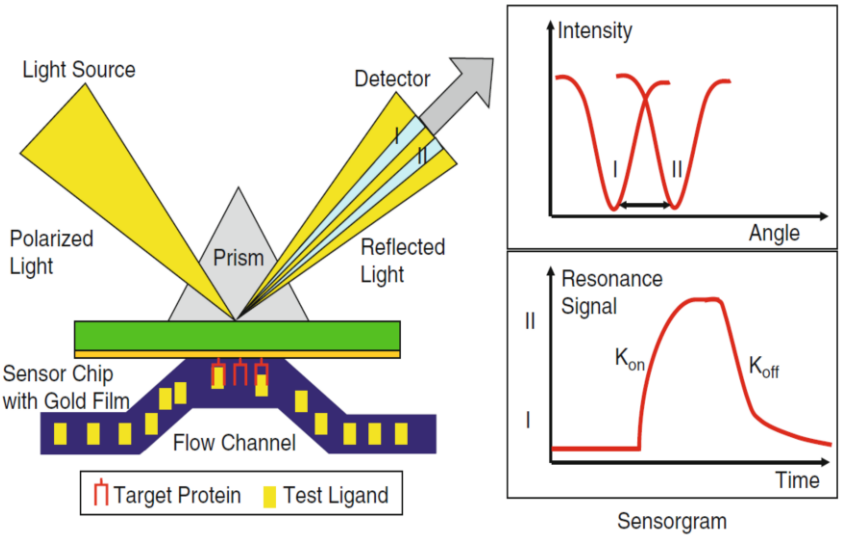


Figure 14. The principle of Surface plasmon resonance (SPR) experiment (62)

Assay Details

This assay was performed using a Biacore®T100 (Biacore, GE Healthcare) instrument. The system was primed twice with running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20). Using a standard amino coupling method, human topo IIα ATPase was immobilized on the second flow cell of a CM5 sensor chip. The topo IIα ATPase domain (fragment containing residues 1–453) was purchased from Inspiralis. The carboxymethylated dextran layer was activated with a 7 min pulse of EDC (1-ethyl-3-(3-dimethylethylaminopropyl)-carbodiimide) and NHS (N-hydroxysuccinimide) in a 1:1 ratio. Human topo IIα ATPase, diluted to a final concentration of 50 mg/mL in 10 mM sodium acetate (pH 5.5), was injected in two short pulses to reach a final immobilization level around 7 000 response units. The rest of the surface was deactivated with a 7 min injection of ethanolamine. The system was reprimed with new running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20). Analytes were prepared as 50 mM stock solutions in DMSO and were diluted with running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) The analytes were injected with concentrations: 6.25, 12.5, 50, 100, 200, 400 µM. To diminish the difference in the refractive index between samples and running

buffer for the titration of analytes, 0.8 % (v/v) DMSO was added to the running buffer. Sensorgrams were recorded.

We tested binding of compounds: **2, 5, 7, 8** and **9**. We did not tested compounds that were not rendered as inhibitors of human topo IIα: **1, 3, 4, 6.** Fits were acceptable with fitting method: two state reaction and were calculated the KD values.

5. Results and Discussion

5.1 Results of the topo IIα HTS relaxation assay

The microplate based HTS topo IIα inhibition assay was performed for the initial screening of the flavonoid hit compounds **1**-**9** to determine if they were able to inhibit the normal process of enzyme relaxation of the supercoiled plasmid catalysed by topo IIα. The structures of the hit compounds are presented in **Table 2**.

The natural hit compounds were supplied in the scope of collaboration between the National Institute of Chemistry in Ljubljana and AnalytiCon Discovery GmbH (Germany). This company is specialized in natural product (NP) drug discovery. All compounds had the general chemical structure of Flavonoids natural produces, comprised of a 15-carbon skeleton, which consists of two phenyl rings (A and B) and a heterocyclic ring (C).

By using the obtained inhibition curves of the measured relaxation activity at different concentrations of the investigated compounds depicted in **Figure 15** IC_{50} values were subsequently calculated for the active compounds and are presented in Table 2.

By analysing the obtained results**,** we were able to conclude that four hit flavonoid compounds **1**, **3**, **4** and **6** were not able to inhibit the enzyme while the remaining 5 hit compounds - **2**, **5**, **7**, **8** and **9** showed promising inhibition activities in the HTS relaxation assay.

To validate the used inhibition assay Etoposide, a clinically used anticancer drug from the group of topo II poisons, was used as a control compound and we experimentally determined the IC_{50} value of 73.0 μM, which was fully comparable to the IC_{50} of 60.3 μM reported in the literature (63).

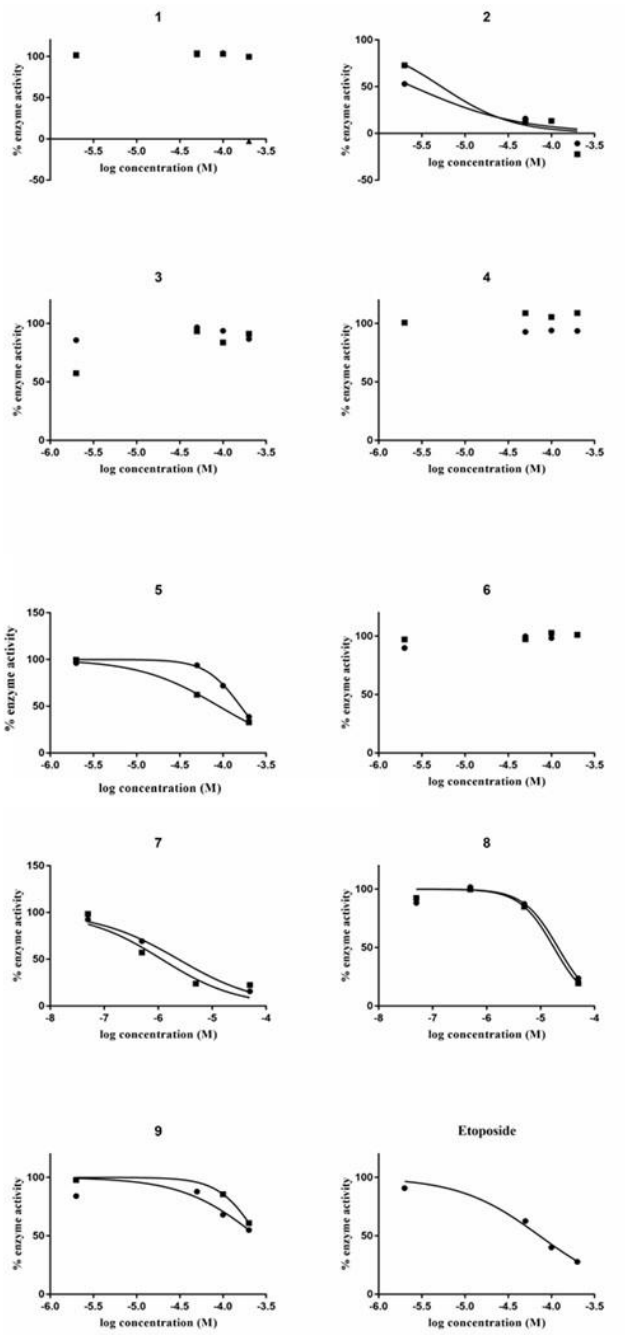
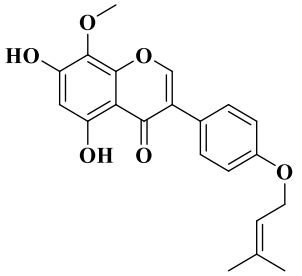
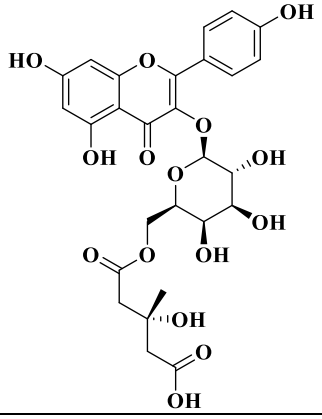
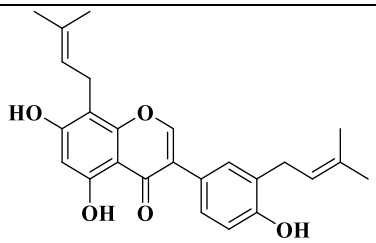
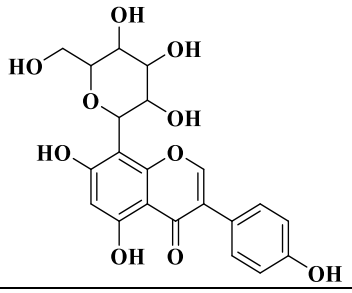
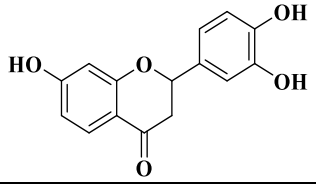
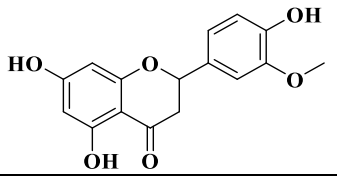
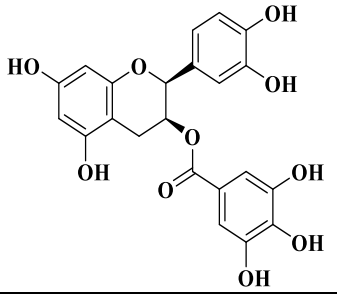
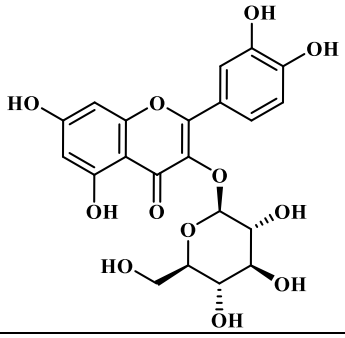
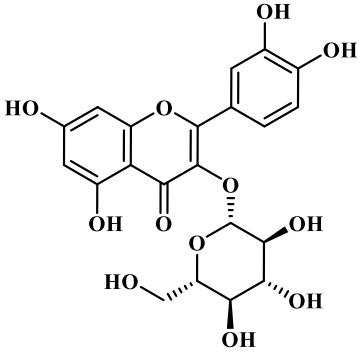
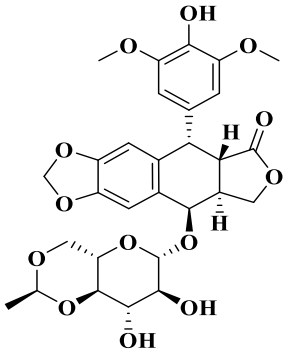


Figure 15 Results of the topo IIα relaxation assay for the investigated flavonoid hit compounds 1-9. The experiment was performed in two independent replicates

Table 2. List of the flavonoid hit compounds discovered by the pharmacophore-based virtual screening and determined IC_{50} values in the topo II HTS relaxation assay

Compound	Chemical structure	Chemical name	IC_{50} (μM)*
1		5,7-dihydroxy-8-methoxy-3-(4-((3-methylbut-2-en-1-yl)oxy)phenyl)-4*H*-chromen-4-one	No inhibition
2		(*R*)-5-(((*2R,3R,4S,5R,6S*)-6-((5,7-dihydroxy-2-(4-hydroxyphenyl)-4-oxo-4*H*-chromen-3-yl)oxy)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methoxy)-3-hydroxy-3-methyl-5-oxopentanoic acid	3.9
3		5,7-dihydroxy-3-(4-hydroxy-3-(3-methylbut-2-en-1-yl)phenyl)-8-(3-methylbut-2-en-1-yl)-4*H*-chromen-4-one	No inhibition
4		5,7-dihydroxy-3-(4-hydroxyphenyl)-8-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)-4*H*-chromen-4-one	No inhibition
5		2-(3,4-dihydroxyphenyl)-7-hydroxychroman-4-one	124.7

6		5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)chroman-4-one	No inhibition
7		(*2S,3S*)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxychroman-3-yl 3,4,5-trihydroxybenzoate	1.75
8		2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-(((*2R,3S,4R,5S,6S*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-4*H*-chromen-4-one	19.4
9		2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-(((*2R,3S,4R,5S,6S*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-4*H*-chromen-4-one	247.9
Etoposide		4'-Demethyl epipodophyllotoxin	73.0

*the IC_{50} values are an average of two measurements

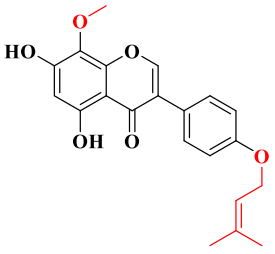
Among the active compounds **5** had a high IC_{50} value (IC_{50} = 124.7 μM). As we browse further through the assays results, we can see that compound **8** (IC_{50} = 19.4 μM) is about 10-fold better inhibitor in comparison to the structurally similar compound **9** (IC_{50} = 247.9 μM),

Compounds **2** (IC_{50} = 3.9 μM) and **7** (IC_{50} = 1.75 μM) were found to be the most potent in the assay displaying the lowest IC_{50} values. As we can see many compounds showed substantially superior inhibition to etoposide.

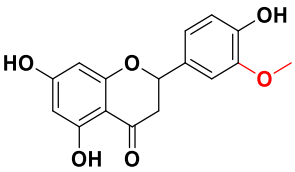
5.2. Initial structure-activity relationship (SAR) of the investigated flavonoid compounds

After obtaining the HTS inhibition results. we then analysed the 2D structures of these compounds to derive the initial Structure–Activity Relationship (SAR) of the flavonoid compounds, before proceeding with the 3D molecular docking calculations.

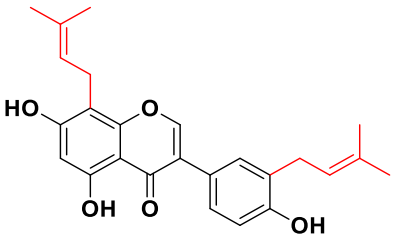
Compounds **1**, **6** and **3** have the same main flavonoid core but the substituent groups marked in red seem to make them **non-inhibitors**, either because of the unfavorability of the methoxy group or the alkyl groups.



1

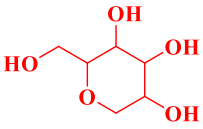


6

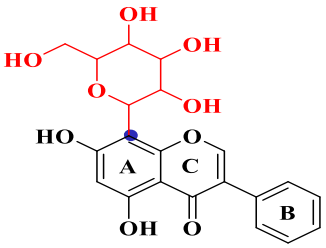


3

If we compare these compounds to most of the active compounds, we can observe that they all lack the sugar moiety, present in most of the active compounds. However, even though compound **4** contained the sugar group, it was still rendered inactive. It could be speculated that this is due to the fact that compound **4** does not have this group at the same position as the other active compounds, namely C ring of the flavonoid core scaffold, but instead is located on the A ring.

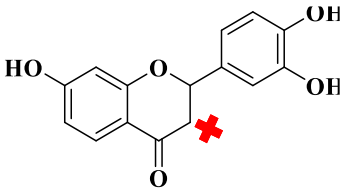


Sugar moiety

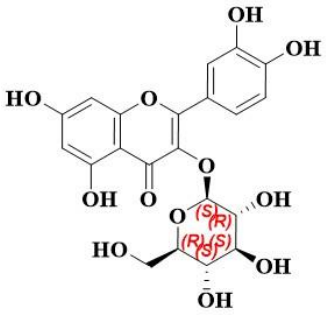


4

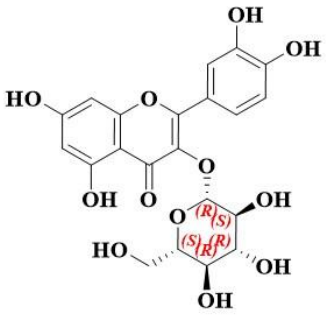
Hit compounds with numbers **2**, **5**, **7**, **8**, **9** displayed the ability of inhibiting the topo IIα enzyme. Among them flavonoid compound **5** does not contain the sugar group (or its mimetic), but has, in comparison with compound **1** or **6,** both hydroxylic groups free. This is a potential reason for the observed inhibition activity of compound **5**.



Active compounds **8** and **9** both have different stereochemistry of the containing sugar moiety: In compound **8** the sugar moiety possesses the configuration (S/R/S/S/R), and in compound **9** (R/S/R/R/S) configuration of the chiral centres is present. This structural change might possess some important characteristic in terms of inhibitory activity since we noticed a ten-fold difference in the inhibitory activity between the two compounds.

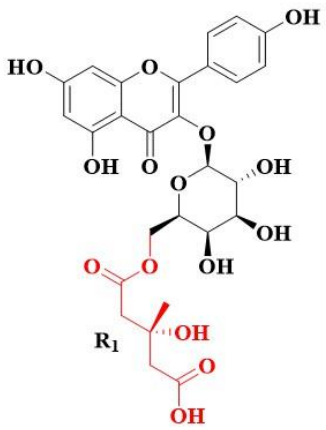


8

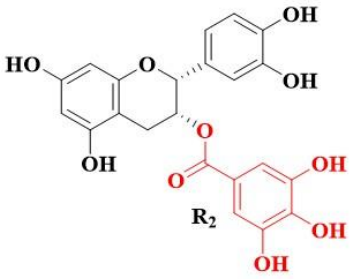


9

Finally, the most active compounds **2** and **7** detected in the HTS assay contained dissimilar moieties **R_1, R_2** attached to the flavonoid core which might also have some important influences on the inhibitory activity.



2



7

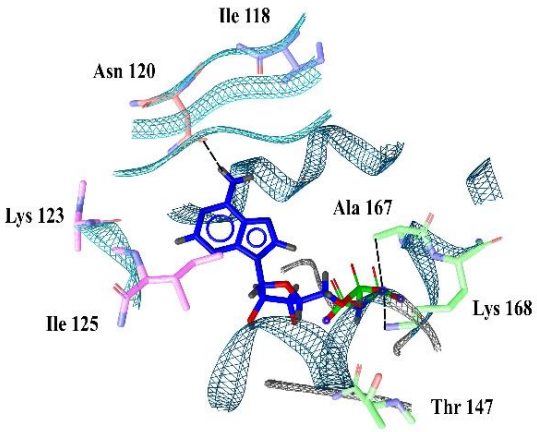
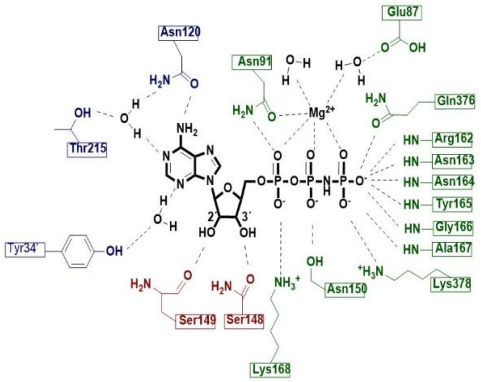
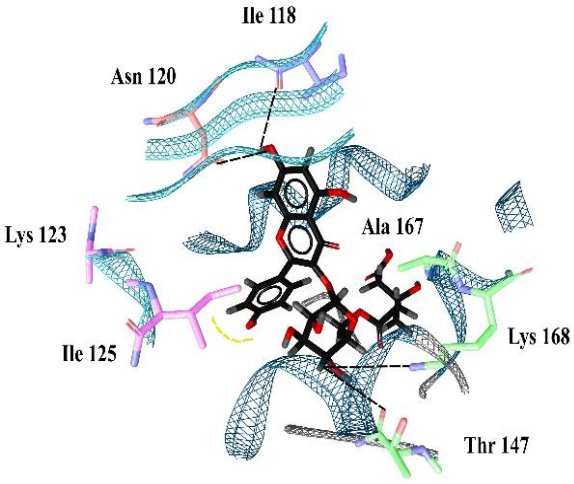
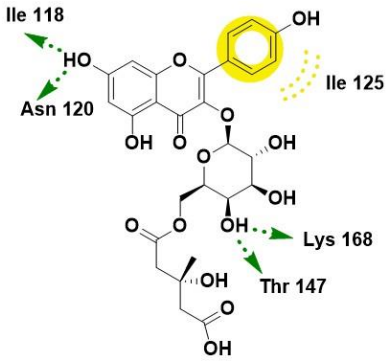
The **R1** moiety comprises an additional hydroxy-containing carboxylic acid attached to the sugar moiety. In the case of the **R2** substituent we concluded that also planar aromatic moieties containing the hydroxylic groups can be an adequate substitute for the sugar moiety leading to the inhibitory activity. Due to the comparable inhibition activities we can conclude that both moieties contribute favourable to topo IIα inhibition

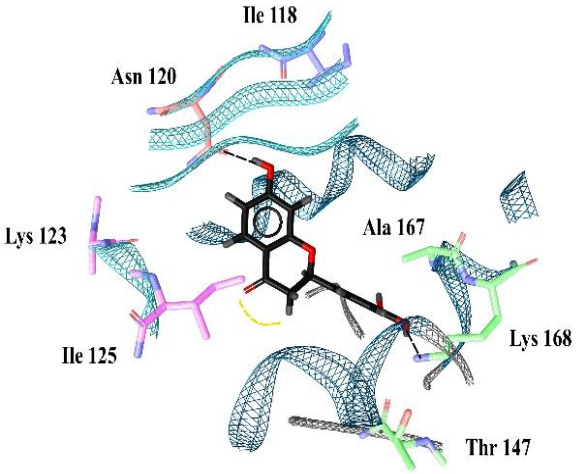
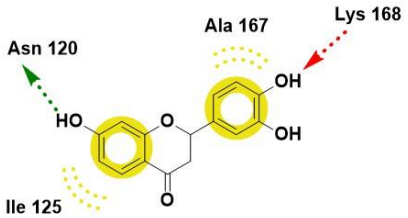
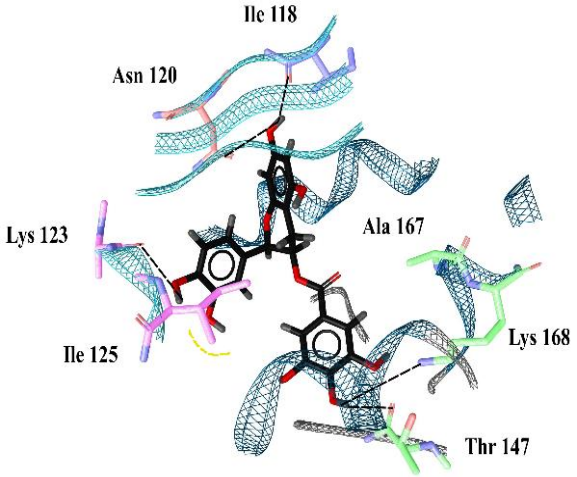
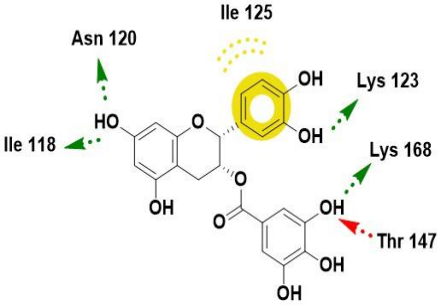
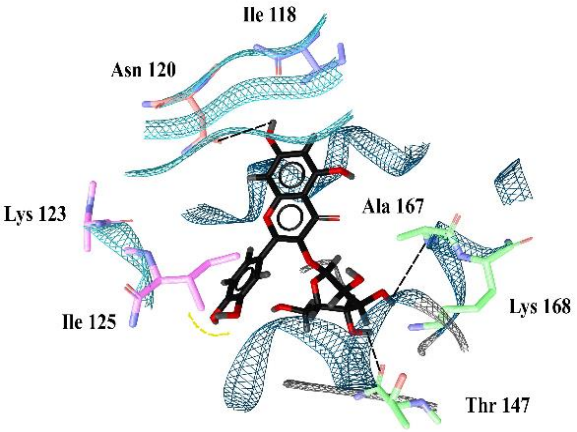
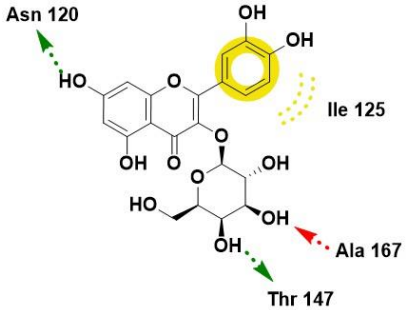
5.3. Molecular docking of active compounds into the ATP binding site

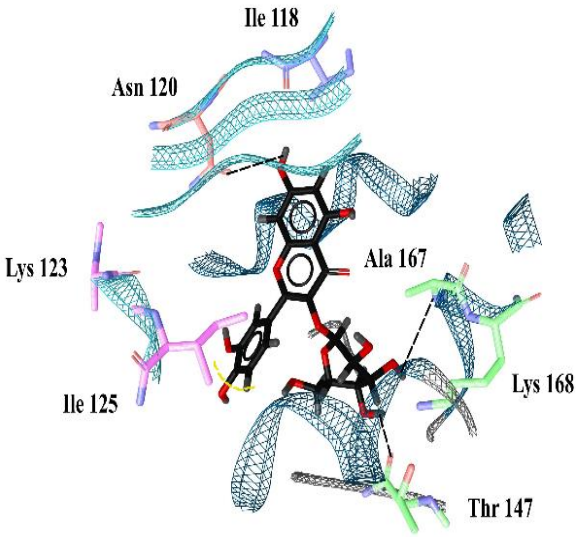
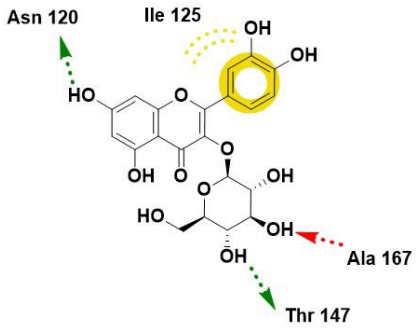
We then docked all active flavonoid compounds **2**, **5**, **7**, **8** and **9** into the crystal structure of the topo IIα ATPase domain (1ZXM), focussing on the ATP binding site, using GOLD molecular docking programme.

All calculated 3D binding poses were kept, and we visually examined them. The representative binding modes for each compound are presented in **Table 3**, along with the derived 2D interaction schemes of the 3D structure-based pharmacophores generated by LigandScout for the docked conformations. The conformation of the native AMP-PNP ligand is included in Table 3 for critical comparison.

Table 3. Docked conformations of the active flavonoid compounds 2, 5, 7, 8 and 9 in the ATP binding site of the human topo IIα and subsequently derived 2D interaction scheme of the 3D structure-based pharmacophores generated by LigandScout for the docked conformations

N	Docking binding mode	3D Structure-based pharmacophore
AMP-PNP		
2		

5	 Ile 118 Asn 120 Lys 123 Ala 167 Ile 125 Lys 168 Thr 147	 Asn 120 Ala 167 Lys 168 HO OH OH Ile 125
7	 Ile 118 Asn 120 Lys 123 Ala 167 Ile 125 Lys 168 Thr 147	 Ile 125 Asn 120 Lys 123 Ile 118 Lys 168 Thr 147
8	 Ile 118 Asn 120 Lys 123 Ala 167 Ile 125 Lys 168 Thr 147	 Asn 120 Ile 125 Ala 167 Thr 147

9		

After molecular docking procedure, the obtained binding geometries derived 3D pharmacophore molecular recognition patterns, hydrogen bond distances between the heavy atoms of the docked ligands and amino acid residues were analysed in light of the experimental inhibition data.

At the first glance all compounds shared a H-bond interaction with Asn120 that mimics the interaction between the adenine moiety of the ATP and the topo IIα ATP binding site. In addition, these compounds were found to possess a hydrophobic interaction between the phenyl ring attached to the bicyclic flavonoid core and Ile125 residue. Only in the case of the active flavonoid compound **5** this phenyl ring formed such similar hydrophobic interaction via the Ala167 residue. Furthermore, a rich hydrogen bond network observed between the residues Ile118, Lys123, Ala167, Lys168 and Thr147 to name a few and topo IIα ATP binding site was predicted to be important for binding (see Table 3 for more detailed visualization of the individual binding modes).

By analysing the molecular recognition patterns of the docked conformations of the active flavonoid compounds **2, 5, 7, 8** and **9** in the topo IIα ATP binding site, it was possible to speculate which structural features could provide some explanation to correlate the IC_{50} values and its 3D structures.

To start with, compound **2** and **7** comprised the most potent discovered inhibitors of this series of natural compounds with the IC_{50} values of 3.9 μM and 1.75 μM respectively. This means that they can inhibit the enzyme at very low concentrations. Their docking binding modes exhibited two H-bond interactions of the core hydroxyl group located on the benzo ring A of the flavonoid main structure, with Asn120 and Ile118 residues. Next, the aromatic ring B attached to the core bicyclic structure formed hydrophobic interaction with Ile125 and finally, the sugar moiety interacted with Thr147 and Lys168 residues.

Active flavonoid compound **8** had a bit higher IC_{50} value of 19.4 μM. This could be attributed to be the fact that it lacks one H-bond interaction (Ile118) of the benzo core ring A hydroxylic group compared to compounds **2** and **7**. Also, the containing sugar moiety doesn't form interactions with Lys168 but instead with the Ala167 residue.

Finally, the remaining two active flavonoids **5** and **9**, exhibited the IC_{50} values of 124.7 μM and 247.9 μM, respectively. Despite the fact that compounds **8** and **9** are structurally very similar, their IC_{50} values diverge for about 10-fold. Most probably this can be attributed to different placement of the hydroxylic groups of the sugar moiety. However, purely our docking results could not detect any significant change.

Lower activity of compound **5** can be explained first by the lack of one H-bond interaction with benzo ring A (Ile118) as well as loss of the interactions enabled by the sugar or "sugar mimetic" moiety present in other molecules.

At the end of this brief analysis it should be emphasised that all these predicted binding modes are a subject an important caveat since no crystal structure between the small molecule inhibitor and topo II ATP binding site is known. We hope that in the future structural data will provide more clues about the actual binding properties of this class of molecules.

5.4. Investigation of the topo IIα inhibition mechanism

Following the HTS topo IIα relaxation assay, we investigated the active compounds inhibitory mechanism in more detail. This is an important step of topo IIα inhibitors

evaluation since, due to the complex catalytic cycle only such assay can provide key information about how these compounds act on the molecular level.

5.4.1 Topo IIα-mediated DNA decatenation assay

To explore further how selected compounds **2** and **7** which were rendered as the most potent in the HTS relaxation assay inhibit the catalytic activity of the enzyme, topo IIα-mediated decatenation assay was performed in collaboration with Inspiralis (Norwich, UK). Etoposide was used as a control compound. The results are presented in **Figure 16**.

This type of assay also enables a direct visual inspection of the inhibition process and is in this respect superior to the HTS assay where the inhibition is detected indirectly via a spectroscopic detection of the formed triplex structure. In addition, in such assays compounds that intrinsically show substantial fluorescence could also interfere with the assay.

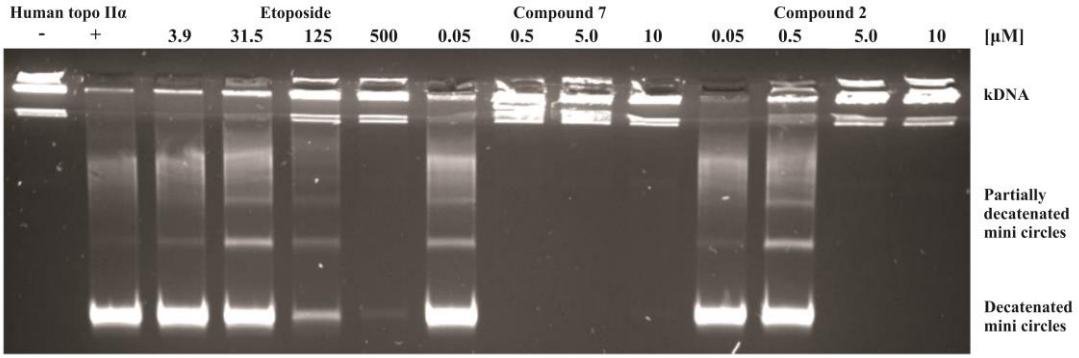


Figure 16. Results of the Human Topo IIα Decatenation assay

First, we analysed the behaviour of the etoposide control active compound where almost no topo II decatenation activity was observed in the presence of **125** and **500** μM concentration of both compounds. Moderate enzyme activity was observed at the **31.5** μM etoposide concentration, placing the IC_{50} value in the expected range between **31.5** μM and **125** μM for this process.

Both flavonoid compounds **2** and **7** inhibited the decatenation of kDNA in a concentration-dependent manner (see also **Table 4** for more details) and even exhibited

significantly higher inhibitory activity compared to etoposide. They showed complete inhibition of the topo II catalysed decantation reaction at as low concentration as **0.5** μM for compound **7** and **5.0** μM for compound **2.**

This assay reconfirmed the inhibitory potential of such compounds by visualizing the DNA topology modifying process catalysed by topo II on the gel and not just measuring it indirectly via fluorescence as it was the case in HTS relaxation assay.

Table 4 Detailed data of the DNA decatenation assay at different concentrations of the investigated compounds 2 and 7

Compound	**% Decatenated Assay 1**				**% Decatenated Assay 2**				**% Decatenated Average**			
Concentration (μM)	**3.9**	**31.5**	**125**	**500**	**3.9**	**31.5**	**125**	**500**	**3.9**	**31.5**	**125**	**500**
Etoposide	100.0	92.4	28.9	4.9	99.9	87.4	25.9	5.2	100	89.9	27.4	5.0
Concentration (μM)	**0.05**	**0.5**	**5.0**	**10**	**0.05**	**0.5**	**5.0**	**10**	**0.05**	**0.5**	**5.0**	**10**
Compound 2	98.0	98.6	0	0	99.2	89.1	0	0	98.6	93.9	0	0
Concentration (μM)	**0.05**	**0.5**	**5.0**	**10**	**0.05**	**0.5**	**5.0**	**10**	**0.05**	**0.5**	**5.0**	**10**
Compound 7	41.1	0.15	0	0	88.3	0	0	0	64.7	0.08	0	0

5.4.2 Topo IIα-mediated Cleavage assay

In order to determine whether our compounds act as topo II poisons, cleavage assay was performed, also in collaboration with Inspiralis (Norwich, UK), using the most potent compounds **2** and **7**. The known topoisomerase IIα poison Etoposide was used as a control compound, as shown in **Figure 17** and **Table 5**.

Topoisomerase II poisons stabilize the transient covalent cleavage complex between the enzyme and DNA and increase its concentration, thus the concentration of linear (cleaved) plasmid is increasing, which this assay can detect; as a rapid denaturation of the topo II prevents the plasmid from resealing and with gel electrophoresis we can discern between the different plasmid form.

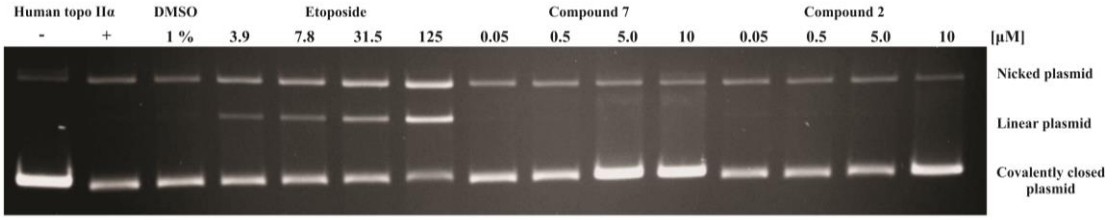


Figure 17. Gel Image: Human topo IIα cleavage assay

From the results it can be seen, that in case of the topo II poison (etoposide) there are higher levels of the linear plasmid visible on the gel with the increasing concentrations of the reference compound. The results thus clearly showed the topo IIα poison activity of etoposide with an increase in linear DNA with increased concentration of the drug. On the other hand, the increased concentrations of compound **2** and **7** did not result in any significantly increased levels of linear DNA above that of background (DMSO alone) level. Thus, the discovered flavonoids cannot be considered as topo IIα poisons but as catalytic inhibitors which is in accordance with the designed mode of action.

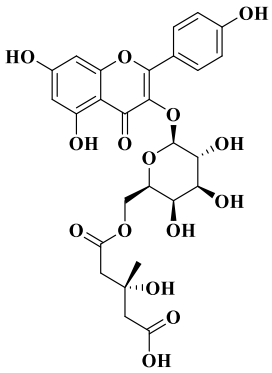
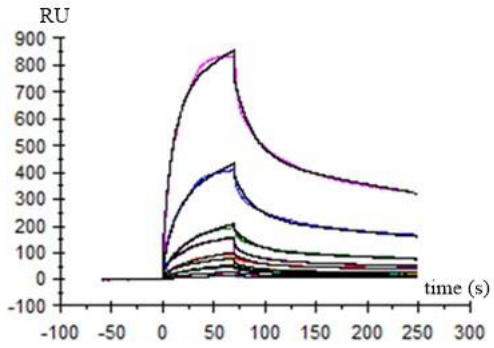
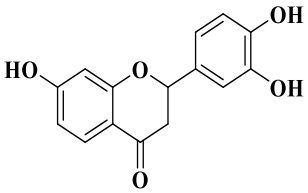
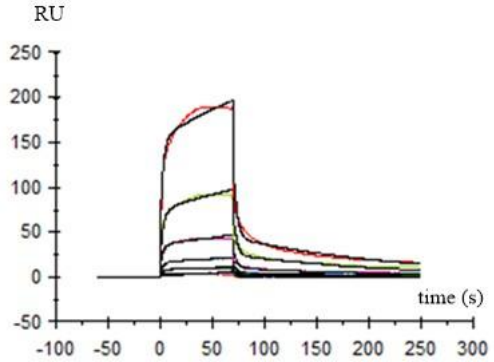
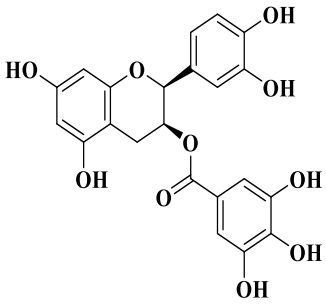
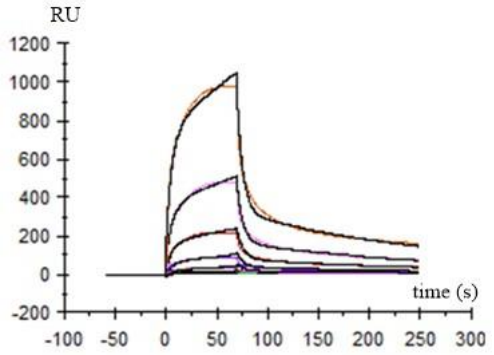
Table 5. Results of the Cleavage Assay

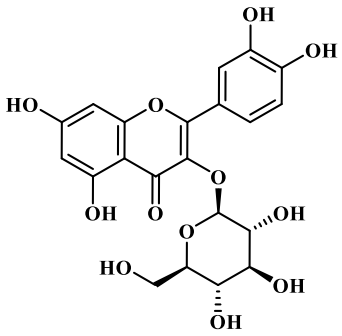
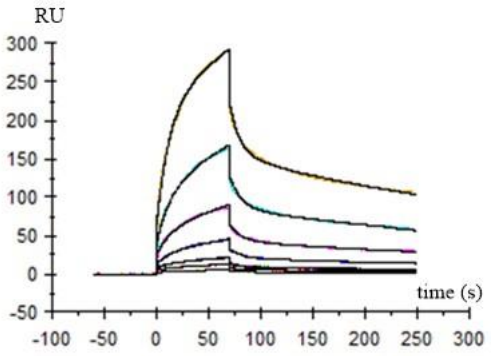
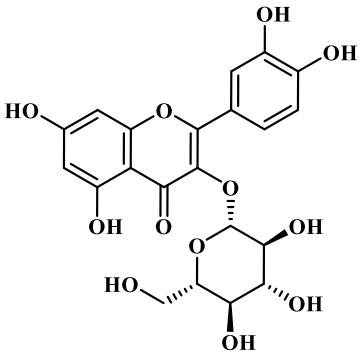
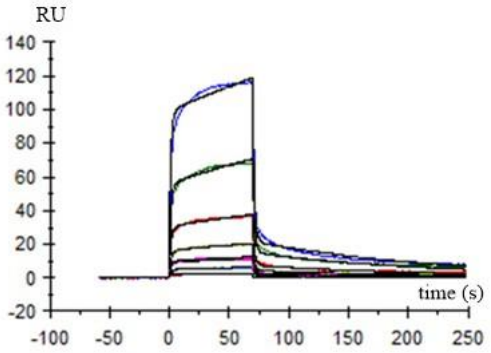
Compound	% Linear Assay 1	% Linear Assay 2	% Linear Average
DNA alone	0	0	**0**
DNA + topo II	0	0	**0**
DNA + topo II + DMSO	0	1.80	**0.90**
Etoposide 3.9 µM	10.31	5.52	**7.92**
Etoposide 7.8 µM	13.40	10.07	**11.73**
Etoposide 31.5 µM	21.43	16.90	**19.16**
Etoposide 125 µM	35.87	31.47	**33.67**
Compound 2 0.05 µM	0	0	**0**
Compound 2 0.5 µM	0	0	**0**
Compound 2 5.0 µM	0	0	**0**
Compound 2 10 µM	0	0	**0**
Compound 7 0.05 µM	0	0	**0**
Compound 7 0.5 µM	0	0	**0**
Compound 7 5.0 µM	0	0	**0**
Compound 7 10 µM	0	0	**0**

5.4.3 Binding studies to the isolated N-terminal domain using SPR technique

Subsequently we investigated if the detected inhibitory activity of the active flavonoid compounds **2**, **5**, **7**, **8** and **9 -** the five compounds who had showed to be inhibitors - was associated with their binding to the isolated ATPase domain using SPR analysis. We used the isolated ATPase domain of human topo IIα since it contains only the ATP binding site. For this purpose, this isolated domain of human topo IIα was immobilized on the CM5 chip. The extent of the changes on the gold surface that are caused by the binding of the substrate ligand molecule onto an anchored target lead to a shift in the resonance angle of the reflected light. In such a way, it is possible to study the association and dissociation activity of the ligand. The real time obtained SPR sensorgrams and the calculated K_D values for the investigated compounds are shown in **Table 6.**

Table 6 Obtained SPR sensorgrams and calculated K_D values obtained using a two-state reaction model

N	Compounds structure	SPR sensorgrams	K_D
2			52.6 µM
5			3.8 mM
7			5.5 mM

8			96.9 µM
9			2.9 mM

By looking at the results, we observed that a nonspecific binding happens at higher concentrations of almost all compounds. This can be associated with different behaviour of the compounds at higher concentration.

To quantitate this behaviour further R*max* theoretical values were calculated using the equation provided bellow:

$$R_{\max theoretical} = \frac{Analyte\ MW}{Ligand\ MW} \times immobilized\ amount\ of\ ligand \times\ stoichiometric\ ratio$$

The results for the investigated compounds were as follows:

$$R_{\max theorical\ Compound\ \mathbf{2}} = \frac{592.5\ g/mol}{54000\ g/mol} \times 6400 \times\ 1 = \mathbf{70.2\ RU}$$

$$R_{\max theorical\ Compound\ \mathbf{5}} = \frac{272.26\ g/mol}{54000\ g/mol} \times 6400 \times\ 1 = \mathbf{32.3\ RU}$$

$$R_{\max theorical\ Compound\ \mathbf{7}} = \frac{442.38\ g/mol}{54000\ g/mol} \times 6400 \times\ 1 = \mathbf{52.4\ RU}$$

$$R_{\max theorical\ Compound\ \mathbf{8}} = \frac{464.38\ g/mol}{54000\ g/mol} \times 6400 \times\ 1 = \mathbf{55.0\ RU}$$

$$R_{\max theorical\ Compound\ \mathbf{9}} = \frac{464.38\ g/mol}{54000\ g/mol} \times 6400 \times\ 1 = \mathbf{55.0\ RU}$$

From the comparison of the calculated R*max* theoretical values with the actually obtained RU values it is clear, that at higher concentrations of all compounds non-specific binding to the investigated ATPase protein occurs. Measured responses are in general between 2 to 19 times higher as theoretical, (compound **9** and **7** respectively) which indicates, that stochiometric ratio for binding at higher concentrations is not 1:1 (inhibitor: protein).

For fitting of our data to obtain the SPR binding curves we used a two-state reaction model, which provided the best fitting of the obtained results and subsequently K_D parameters were calculated. The two-state reaction model describes binding of the ligand to immobilized target followed by a conformational change that stabilizes the complex. The K_D values obtained by this analysis were quite high compared IC_{50} values. The best binding was observed for compounds 2 and **8**, where the K_D values (K_D = 52.6 µM and K_D = 96.9 µM) was somewhat comparable with the determined IC_{50} values of 3.9 and 19.4 µM respectively.

Overall the SPR experiments conformed that flavonoid compounds do interact with the ATPase domain where the targeted ATP binding site is located. However, due to the ambiguity of the SPR results further studies are needed such as for example kinetic studies to fully establish if compounds do indeed act via the targeted mechanism of ATP inhibition. The researchers at National Institute of Chemistry and at Infrastructural Centre for Molecular Interactions Analysis in Ljubljana will evaluate this aspect in the near future.

6. Conclusions

The human DNA topoisomerase IIα is an essential enzyme that plays a key role in cell's replication machinery and is an established anti-cancer target with several inhibitors already in clinical use. Due to the known limitations imposed by severe side effects and cancer cell resistance associated with the established group of clinically used topo II poisons, new paradigms of topo II inhibition are being actively pursuit among them compounds that target the ATP binding site of this DNA topology modifying molecular motor.

We started our research from a previously performed large-scale pharmacophore-based virtual screening of a library of natural products that identified nine natural compounds from the flavonoid chemical class, with potential ability to inhibit the topo IIα. By using the HTS relaxation assay, we determined that five out of nine virtual hit compounds - flavonoids 2, 5, 7, 8 and 9 – possessed topo II inhibition ability in the low micromolar range. Most compounds were more active than the etoposide reference topo II drug.

Following this promising experimental data, the initial 2D-based SAR was established for these natural products outlining functional groups and substituents that can contribute favourably or unfavourably to the ligand´s binding free energy Succeeding molecular docking calculations showed that all active compounds shared a H-bond interaction with Asn120 residue that mimics the experimental interaction between the adenine moiety of the ATP and the topo IIα ATP binding site. In addition, active flavonoids displayed a hydrophobic interaction between the phenyl ring attached to the bicyclic flavonoid core and a hydrophobic residue, in most cases, the Ile125 residue.

Due to the complex catalytic cycle associated with topo IIα mode of action, we then performed further experiments to probe the inhibition mechanism of the investigated compounds. With the topo IIα mediated cleavage assay, we showed that compounds 2 and 7 act as catalytic inhibitors. Moreover, with the topo IIα mediated decatenation assay, we proved with the application of an alternative assay that these two compounds do inhibit the enzyme at much lower concentrations than the reference drug etoposide.

Finally, the SPR measurements confirmed that active flavonoids do indeed bind to the isolated ATPase domain of the human topo IIα. The best fitting was achieved using a two-state model, which describes binding of the ligand to the studied targeted truncated protein which is followed by a conformational change that stabilizes the complex. Due to the ambiguity of the SPR results, further studies will be needed to establish if compounds do indeed act via sorely the ATP competitive mechanism or if the inhibition mode is even more complex.

The obtained results could potentially provide new information about these lead compounds of the natural origin –flavonoids- that could pave the way to further drug development leading to novel anticancer agents exploiting the catalytic topo IIα inhibition paradigm.

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